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The effect of systemically and locally administered clodronate on bone quality

by

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ACADEMIC DISSERTATION

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Abstract

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The aims of this study were to evaluate the effect of clodronate, given systemically or locally, on the quality of bone and to develop a combination product including clodronate and bioactive glass for local administration in the treatment of periodontitis.

The beneficial effects of clodronate on bone are known. Clodronate inhibits osteoclasts in bone and reduces bone turnover. It is used in breast cancer patients with non-osseous metastases to reduce the osteolytic complications and bone metastases. It is widely investigated e.g., in healthy women reducing bone loss. The first aim of this thesis was to investigate whether these established effects are similar in women with primary operable breast cancer.

Since skeletal bone loss and alveolar bone loss in periodontitis share common mechanisms, the effect of clodronate upon dental application was of interest. Systemic bone conditions impact the periodontium and systemically administered clodronate positively affects the periodontium, but also side effects such as diarrhea, rash and osteonecrosis of the jaw can be problematic. However, clodronate is poorly absorbed from gastrointestinal (GI) tract and the oral bioavailability of clodronate is low. Therefore, systemically administered oral dose of clodronate needs to be high to achieve a therapeutic effect, which in turn leads to increased adverse events. Therefore, the challenge in developing novel drug delivery systems for clodronate is to achieve improved bioavailability and safety. The aim of the second part of this thesis was to develop a new delivery system that would reach the target site in the periodontium, while limiting unwanted side effects and reducing the required dose through local administration.

In the first part of this study, the loss of bone mineral density (BMD) was studied in patients with primary operable breast cancer given either clodronate or placebo. Oral clodronate appears to reduce the loss of bone in these patients. In premenopausal patients clodronate significantly reduced the loss of bone after one year and in postmenopausal patients clodronate increased the spinal BMD. Patients receiving clodronate had significantly more incidences of diarrhea than those receiving placebo. This indicates that clodronate is poorly absorbed from GI tract thus causing irritation to the intestine. In addition, the development of bone metastases was compared with patients with primary operable breast cancer given either clodronate or placebo. Clodronate given to these patients was shown to reduce the occurrence of bone metastases. Additionally, there was a significant reduction in mortality.

In the second part of the study, a novel combination product of bioactive glass (BAG, SiO₂ 53%, Na₂O 23%, CaO 20% and P₂O₅ 4% (w/w %)) and clodronate was investigated. Firstly, preformulation studies were performed. Clodronate was found to promote the activity of the BAG and a calcium clodronate precipitate formed. Additionally, the bioactivity lasted longer in the combination product than in BAG alone. The optimal ratio for bioactive glass and clodronate and optimal particle size for BAG for local treatment of periodontitis was investigated and selected based on bioactivity of the BAG, safest pH profile of the combination product, as well as highest possible amount of clodronate to achieve the enhanced bioactivity for the BAG. The combination product chosen was 1 g BAG with a particle size of 0.5-0.8 mm and 200 mg of clodronate premoisturized with saline.

Finally, the selected combination product was compared to the BAG alone in the treatment of the periodontitis maintenance phase in a pilot study with ten study subjects. Based on the clinical signs and symptoms of inflammation or infection at the site (evaluated by the investigator) and overall satisfaction the combination product is at least as good as bioactive glass alone. Of the two bone remodeling biomarkers (osteoprotegerin and osteocalcin) selected for the study, only osteoprotegerin data indicate that the effect of the novel combination product is beneficial. Osteoprotegerin levels decreased in both combination product and BAG alone treated teeth but less in the combination product treated teeth. However, due to the short time period of investigation as well as the limited number of subjects and treated teeth, the result for bone quality remains only indicative. The positive effect of the combination product on tooth sensitivity may bring additional benefits in comparison to the use of BAG alone in periodontal maintenance therapy.

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In Lohja, on the 5th of May, 2014

A handwritten signature in dark ink, appearing to read "Kirsi Rosenqvist". The script is cursive and somewhat stylized, with the first name "Kirsi" and the last name "Rosenqvist" clearly distinguishable.

Kirsi Rosenqvist

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List of original publications

This thesis is based on the following publications, which are referred to in the text by their respective roman numerals (I-V).

- I Powles TJ, McCloskey E, Paterson AHG, Ashley S, Tidy VA, Nevantaus A, **Rosenqvist K**, Kanis J. Oral clodronate and reduction in loss of bone mineral density in women with operable primary breast cancer. J Natl Cancer Inst 1998;90:704-708.
- II Powles T, Paterson S, Kanis JA, McCloskey E, Ashley S, Tidy A, **Rosenqvist K**, Smith I, Otterstad L, Legault S, Pajunen M, Nevantaus A, Männistö E, Suovuori A, Atula S, Nevalainen J, Pylkkänen L. Randomised, placebo-controlled trial of clodronate in patients with primary operable breast cancer. J Clin Oncol 2002;20:3219-3224.
- III **Rosenqvist K**, Airaksinen S, Fraser SJ, Gordon KC, Juppo AM. Interaction of bioactive glass with clodronate. Int J Pharm 2013;452:102-107.
- IV **Rosenqvist K**, Airaksinen S, Vehkamäki M, Juppo AM. Evaluating optimal combination of clodronate and bioactive glass for dental application. Int J Pharm 2014;468:112-120.
- V **Rosenqvist K**, Gürsoy M, Gürsoy U, Könönen E, Juppo AM. Novel bioactive glass – clodronate combination as an adjunctive agent at periodontal maintenance phase. Submitted.

The publications are referred to in the text by their Roman numerals.

Patent related to this study:

Rosenqvist K, Sivéén M, Juppo AM. PCT 2012/No. 20125896: Composition for tissue repair and regeneration

Abbreviations

ADA	American Dental Association
ATR-IR	Attenuated total reflectance infrared
AUC	Area under the curve
BAG	Bioactive glass, S53P4, which is studied in this thesis
BG	Bioactive glass, first generation bioactive glass, so called Hench glass
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenetic protein
BMU	Bone multicellular unit
BP	Bisphosphonate
BOP	Bleeding on probing
BUA	Bone ultrasound attenuate
CaClod.	Calcium clodronate
Clod.	Clodronate
DEXA	Dual energy X-ray adsorption
DNA	Deoxyribonucleic acid
DPA	Dual-photon absorptiometry
DSC	Differential scanning calorimetry
EDS	Energy dispersive X-ray spectroscopic
<i>e.g.</i>	<i>Exempli gratia</i> (for example)
FGF	Fibroblast growth factor
FIB	Focused-ion beam
FTIR	Fourier transform infrared
GCF	Gingival crevicular fluid
GI	Gastrointestinal
HA	Hydroxyapatite
HCA	Hydroxycarbonate apatite
<i>i.e.</i>	<i>Id est</i> (that is)
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
IR	Infrared
ISO	International organization of standardization
M-CSF	Macrophage colony-stimulating factor
MRI	Magnetic resonance imaging
OPG	Osteoprotegerin
PAL	Probing attachment level
PDGF	Platelet-derived growth factor
PPD	Probing pocket depth
PSD	Particle size distribution

PTH	Parathyroid hormone
PTHrP	PTH-related peptide
QCT	Quantitative computed tomography
RANK	Receptor activator of nuclear factor-kappa B
RANKL	Receptor activator of nuclear factor-kappa B ligand
RNA	Ribonucleic acid
SBF	Simulated body fluid
SEM	Scanning electron microscopy
SPA	Single photon absorptiometry
Sv	Sievert
SXA	Single X-ray absorptiometry
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRIS	Tris(hydroxymethyl)aminomethane
UTV	Ultrasound tissue visualization
VAS	Visual analog pain scale
VPI	Visible plaque index
XRPD	X-ray powder diffractometry

1 Introduction

Both clodronate and bioactive glass have been clinically used for decades. Even though much data are available on their use and mechanisms of action, most studies to date indicate that there are still unexplored features and indications of these systems. Clodronate and bioactive glass affect bone, which is an organ with vitally important functions. Therefore, it is interesting to further study these substances and optimize their therapeutic potential in this context.

Bisphosphonates, such as clodronate ($\text{CH}_2\text{Cl}_2\text{O}_6\text{P}_2\text{Na}_2 \cdot 4\text{H}_2\text{O}$, disodium salt form), are agents that inhibit osteoclasts in bone and reduce bone turnover (Fleisch 1997). Bisphosphonates are widely used in breast cancer to treat hypercalcemia (Urwin et al. 1987). In women with metastatic breast cancer, clodronate has been shown to reduce the osteolytic complications of metastases, such as hypercalcemia, bone pain, and vertebral fracture (Paterson et al. 1993). Furthermore, in patients with non-osseous metastases from breast cancer, clodronate can reduce the risk of developing bone metastases (Kanis et al. 1996). In healthy women, bisphosphonates have been shown to be effective in reducing bone loss (Giannini et al. 1993, Filipponi et al. 1995, Liberman et al. 1995). In this study the possibility of similar effects in a different patient population, premenopausal and postmenopausal women who have had primary operable breast cancer (without metastases) and were receiving adjuvant chemotherapy and/or drug treatment, were investigated (**I, II**).

The oral bioavailability of clodronate is very poor and depends on dosage (the absolute mean bioavailability is about 2%) (Villikka et al. 2002). Gastrointestinal absorption of bisphosphonates is low and there is also high intersubject (Saha et al. 1994) and day-to-day within-subject variation (Castren-Kortekangas et al. 1997). Additionally, oral absorption of bisphosphonates is diminished when the drug is given with meals, especially in the presence of calcium and iron (Laitinen et al. 2000). Because of the poor oral bioavailability, the systemically administered oral dosage needs to be high to achieve the therapeutic effect although the appearance of adverse events will increase. All these factors create problems both in research and in clinical use of these drugs. The challenge for novel drug delivery systems is to achieve improved bioavailability and safety, which can potentially be achieved through targeted locally administered formulations (**III, IV, V**).

Skeletal bone loss and alveolar bone loss in periodontitis share common mechanisms. Systemic bone conditions impact the periodontium. Skeletal bone mineral density is reported to be related to interproximal alveolar bone loss and to clinical attachment loss, implicating postmenopausal osteopenia as a risk indicator for periodontal disease (Tezal et al. 2000). Bisphosphonates used for systemic bone loss or as an adjuvant therapy of cancer affect the maxilla and mandible. Systemic bisphosphonate therapy has been reported to have a negative

side effect called osteonecrosis of the jaw (Montazeri et al. 2007). This adverse event is mostly associated with intravenous administration of bisphosphonate and nitrogen containing bisphosphonates (Ruggerio et al. 2004). On the other hand, bisphosphonate therapy is also reported to be beneficial to the periodontium: reduced plaque accumulation, gingival inflammation and periodontal attachment loss, as well as lower probing depths and increased alveolar bone levels have been observed (Shoji et al. 1995, Palomo et al. 2005). Clodronate has been administered locally for the treatment of experimental periodontitis in rats. The results of that study suggest that local administration of clodronate may be effective in preventing osteoclastic bone resorption in periodontitis (Mitsuta et al. 2002). Developing bisphosphonates to slow the progression of periodontal disease depends on identifying an effective dosage regimen and delivery system that would reach the target site locally in the periodontium. At the same time the local administration of clodronate would limit unwanted systemic side effects and the problem of poor gastrointestinal absorption would be avoided. The bioactivity process of bioactive glass (first known as Hench glass, BG) is known to start when it is immersed into biological fluids in vivo, simulated body fluids, or other buffered solutions in vitro. Ion leaching and exchange with surrounding solution results in bone-like apatite layer formation on the BG surface, and a slight alkalization of the surrounding solution is simultaneously induced. These two phenomena form the basis for the osteopductive properties of BG (Hench and Wilson 1984, Kokubo and Takadama 2006). While knowing all this the possibility of using BAG (bioactive glass with composition of S53P4) and clodronate as a combination product in the treatment of periodontitis maintenance phase was of interest (V).

Bones and teeth protect the internal organs, allow enhanced mobility, enable mastication of food, perform other mechanical functions, and are a regulatory source of inorganic ions like calcium, magnesium, and phosphate. In addition, they store many cells (e.g., blood producing precursor cells and adipose cells) and growth factors (Boskey 2007).

In the US alone, approximately 250 000 new breast cancer cases are diagnosed annually. Breast cancer is the second most frequently diagnosed cancer in women after skin cancer. Breast cancer is also the second most deadly form of cancer in women (after lung cancer) with about 40 000 breast cancer deaths yearly in US (American Cancer Society 2013). In Europe, around 460 000 breast cancer cases and 130 000 deaths were estimated in 2012 (Ferlay et al. 2013). The numbers are suggestive, as the way of measuring and defining the cases might differ, but the message is clear. Death rates for breast cancer have steadily decreased in women since 1989, with larger decreases in younger women (<50 years of age) (American Cancer Society 2013). The decrease in breast cancer death rates represents progress not only in earlier detection and improved treatment, but more advanced adjuvant therapies like use of clodronate to prevent secondary diseases.

The risk factors for periodontitis include diabetes, smoking, certain periodontal bacteria, aging, gender, genetic predisposition, systemic diseases and

conditions (like cancer and its treatments), stress, nutrition, pregnancy, HIV infection, substance abuse, and medications (Boskey 2007). Based on this, it is understandable that the prevalence of periodontitis is growing and is in Finland about 64% of population (National Institute of Health and Welfare 2004). Therefore, it is important to take care and protect the quality of our bones and teeth.

The number of patients suffering from all the above mentioned conditions or diseases (e.g. breast cancer, osteoporosis and periodontitis) are known to be growing as our population ages and thus more alternative treatments for these conditions and diseases are needed.

2 Literature overview

2.1 Bone

Bone tissue has two major tasks: to maintain optimal structure to fulfil its mechanical function and to serve as an ion bank taking care of the calcium homeostasis. Macroscopically, bone can be divided into an outer and inner part. The outer part is called cortical or compact bone and the inner part is cancellous *i.e.* trabecular bone. Cortical bone forms about 70%-80% of the skeleton total mass. Microscopically bone can be divided in woven (irregular structure of loosely packed collagen fibrils) and lamellar bone (well-ordered parallel collagen lamellae). In adults only lamellar bone is present both in cortical and cancellous bone. However, in certain conditions such as in fracture healing, when there is rapid bone formation, woven bone is present. In cortical bone, bone cells (osteoblasts, osteoclasts, osteocytes and lining cells) are organized as cylinder structures around blood vessels (Fig. 1).

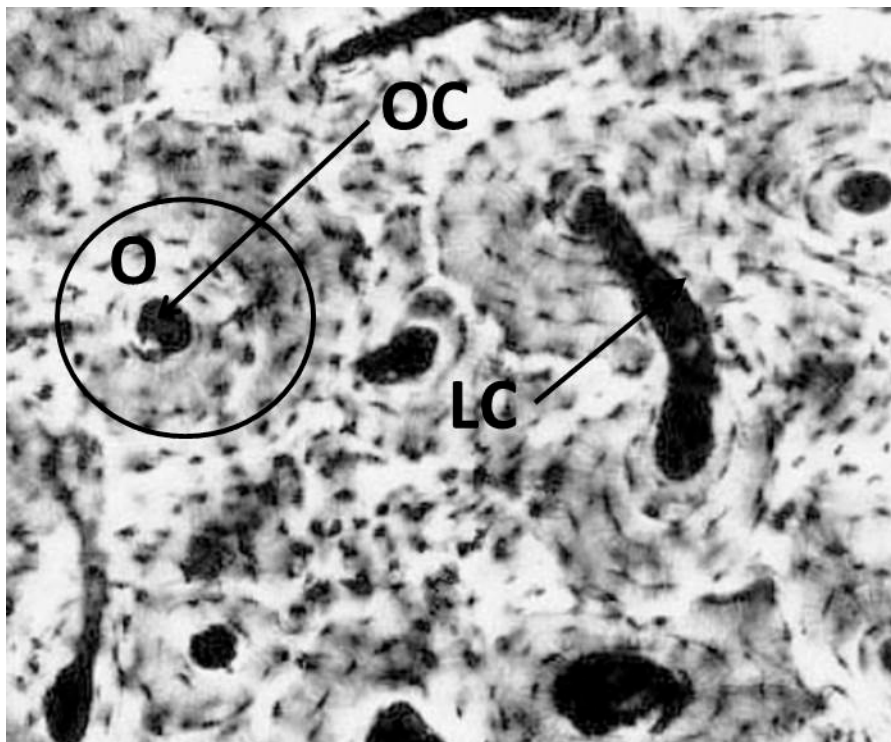


Figure 1. Cross section of compact bone with osteocytes (LC lateral canal, O osteon, OC osteonal chanal).

These multi-cell units are called osteons (Fig. 1). The diameter of an osteon is about 200 μm and various osteons are in communication with each other via canals even if they are separated from one another by cement lines. In the trabecular bone these subunits are called “packets” and if they are on the surface and not yet terminated they are called “bone multicellular units” (BMU). Since trabeculae generally possess no vessels they are nourished from the surface. The trabeculae and inner cortex are the locations that are most affected by osteoporosis (Fleisch 1997).

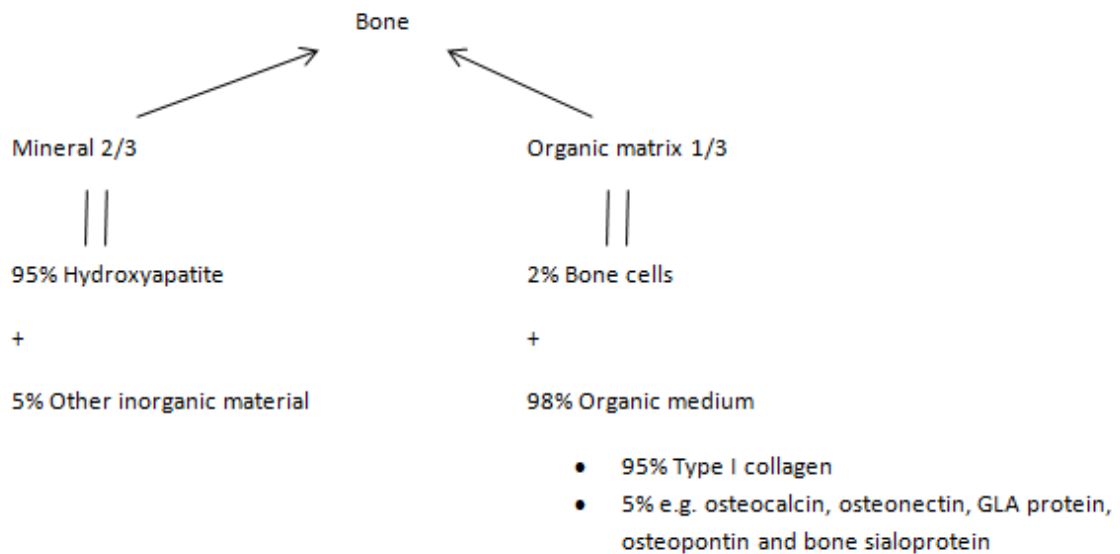


Figure 2. *Composition of bone (dry weight).*

Bone contains minerals, organic matrix, cells and water (1/4 from total bone weight). As seen in Figure 2 the mineral part is about two-thirds of total dry weight of bone. It is basically hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, often referred to generally as calcium phosphate, CaP), which contains many other constituents. Organic matrix is mostly collagen. Hydroxyapatite is crystallized and located within and between the collagen fibers. Turnover and metabolism is rapid in bone tissue. Minerals are constantly exchanged with blood salts.. The bone medium serves as a depot for many growth factors and cytokines (Korhonen and Väänänen 1992, Robey 1995, Fleisch 1997).

After the growth phase the geometry of bones takes its final shape. Periosteal thickening still happens and mineral content of bone increases for couple of years as the peak mass of the bone is achieved (Välimäki et al. 1994). In adulthood changes in bone are mostly related to remodeling of the bone (Fig. 3). The bone

half-life in humans for entire body mass is about 12-15 years. It is longer in cortical bone than in trabecular bone. Bone is renewed in small remodeling units and it involves at least four stages: activation, resorption, recovery and synthesis (Parfitt 1988, Väänänen 1996). Bone remodeling is mediated through bone cells. The bone remodeling unit (BRU) is composed of a tightly coupled group of osteoclasts and osteoblasts that sequentially carry out resorption of old bone and formation of new bone (Clarke 2008). The osteoblasts (derived from mesenchymal stem cells in bone marrow) are the cells that synthesize the bone matrix. There are many hormones and cytokines that influence their function. When osteoblasts are not in the process of forming bone, they are flat shaped and called resting osteoblasts, or, lining cells. Active and resting osteoblasts form a layer at the surface of the bone tissue. At some point the osteoblasts stop synthesizing the organic bone matrix, i.e., osteoid, which is a protein mixture secreted by osteoblasts, and become embedded within bone. They are then called osteocytes. Bone is formed when osteoid mineralizes. The role of a fourth cell type in bone, osteoclasts (derived from granulocyte-macrophage colony-forming unit), is to resorb bone (Väänänen 1996, Fleisch 1997, Clarke 2008).

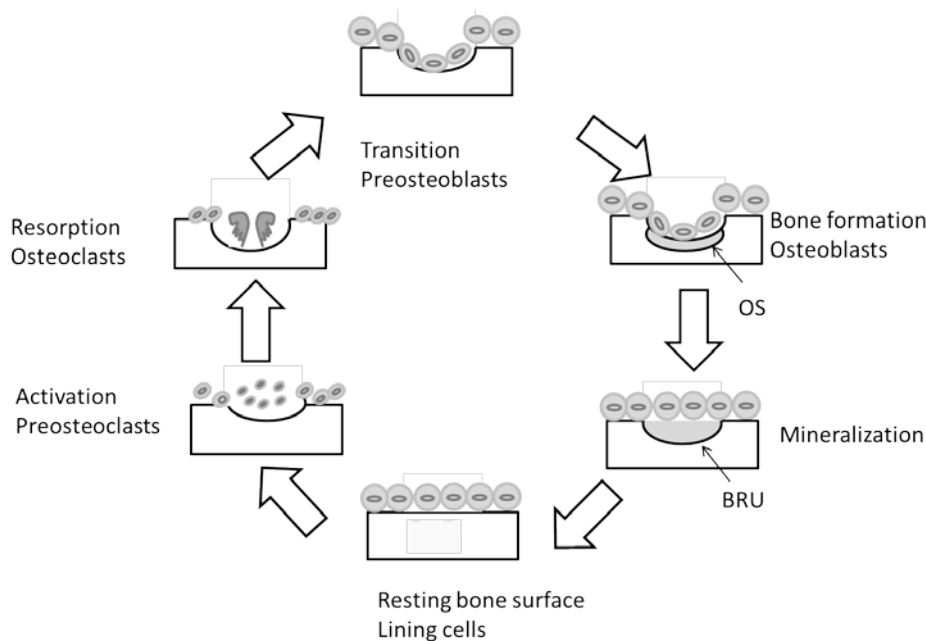


Figure 3. Bone remodeling cycle (*OS*, Osteoid; *BRU*, Bone remodeling unit).

The resorption is performed in a closed microenvironment between the cell and the bone. Bone resorption can be modulated by different mechanisms, altering the recruitment of new osteoclasts, the activity of mature osteoclasts and the number of osteoclasts. All are influenced by several cytokines (e.g. interleukins 1, 3, 6 and 11, tumor necrosis factor) and hormones. In adult bone, remodeling and resorption of the bone are in balance, which results in the replacement of old bone by new bone. The rate of remodeling is between 2% and 10% of the skeletal

mass per year depending on the site and the condition of the bone. Most of the turnover happens in cancellous i.e., trabecular bone. Therefore osteoporosis, which is result from abnormal bone turnover, is first observed in cancellous bone (Väänänen 1996, Fleisch 1997). There are many agents that affect bone formation and resorption, and most of these are listed in Table 1.

Table 1. *Modulators of bone formation and resorption. BMP, bone morphogenetic protein; FGF, fibroblast growth factor; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin, M-CSF, macrophage colony-stimulating factor, PDGF, platelet-derived growth factor; PTH, parathyroid hormone; PTHrP, PTH-related peptide; TGF, transforming growth factor; TNF, tumor necrosis factor.*

Bone formation		Bone resorption	
Increase	Decrease	Increase	Decrease
Systemic			
Fluoride	Corticosteroids	PTH	Calcitonin
PTH		PTHrP	Estrogen
Prostaglandins		Calcitriol	
Cytokines		Thyroxin	
Local			
BMPs	FGFs	IL-1, 6 and 11	TGFβ
TGFβ	PDGFs	FGFs	IFNγ
IGFs	Prostaglandins	Prostaglandins	IL-4
		TNFα and β	
		TGFβ	
		M-CSF	

2.1.1 Effect of breast cancer and cancer treatments on bone

In breast cancer, both the disease itself and the subsequent treatments affect bone. The adverse effects of radiation on tissue are related to damage in three different structures: parenchymal cells, connective tissue and blood vessels. In stroma tissue, fibrosis is a common late reaction caused by radiation. Radiation causes premature differentiation of fibroblasts, which then increases interstitial collagen synthesis and amount. On the other hand, the excretion of certain growth factors (e.g. transforming growth factor β 1) also increases the number of fibroblasts (Kouri and Valavaara 1997). These are the same factors that regulate bone turnover.

Cytotoxicity during chemotherapy is largely restricted to cells that are in the dividing phase. The effect on lining or resting cells is normally minor. The effectiveness of a chemotherapy agent depends on the rate the tumor cells are dividing as well as the amount of those cells. Chemotherapy is based on biochemical reactions and normally the targets in the human cell are deoxyribonucleic acid (DNA), ribonucleic acid (RNA) synthesis and function, proteins (including some enzymes), and membranes. In addition to the intended effect, chemotherapy has also adverse pharmacological effects. In premenopausal women, chemotherapy is known to frequently cause chemical castration, ovarian function failure and premature menopause (Padmanabhan et al. 1987, Richards et al. 1990, Bianco et al. 1991, Saarto et al. 1996, Elonen and Wiklund 1999).

Cancer can also be hormone mediated. This includes administering hormonal therapy that inhibits cancer cell growth, or agents that inhibit endogenous hormones that promote cancer cell growth. Additionally, the production of hormones promoting cancer and cancer cell growth can be totally inhibited. The most commonly administered hormones are estrogen, androgen, progestin and glucocorticoids – all of which also affect bone turnover. Hormone mediated cancer treatment is long-term, and in some cases treatment may be life-long. If hormonal therapy is used as an adjuvant treatment for breast cancer, the treatment time is normally from three to five years (Kataja and Johansson 1999).

Biological cancer treatment is another form of cancer therapy, based on destroying cancer tissue by enhancing the immune system or by using natural agents that destroy cancer cells or limit their growth. Typical agents include cytokines such as interferons, interleukins and hematopoietic growth factors (Kellokumpu-Lehtinen 1999) — once again agents that are involved in bone turnover.

Cancer induced osteolytic bone disease often manifests itself as increased bone resorption and tumor induced osteolysis. In osteolysis, the cancer cells can directly destroy bone or activate bone cells for the same purpose. Osteolysis may be categorized as local and generalized. In localized osteolysis, tumor cells or bone marrow cells around tumor cells excrete transmitter agents (lymphotoxin, tumor necrosis factor, prostaglandins, and interleukins) that locally cause bone destruction, i.e., local osteolysis. In generalized osteolysis, tumor cells or surrounding bone marrow cells excrete transmitters (parathormone like peptide,

calcitriol) into the circulation that promotes bone destruction. Commonly, bone cells (osteoclasts alone or together with osteoblasts) are activated by transmitters to destroy bone. Collagen degradation products can also activate cancer cells by drawing them to the endosteal bone surface, where resorption occurs (Blomqvist 1986, Mundy 1990). Mechanisms of hypercalcemia of malignancy are lytic bone metastases, transmitters increasing bone resorption or combined effect of both. Hypercalcemia occurs in approximately 20% of breast cancer patients (Ritch 1990).

In 1994, the World Health Organization estimated that, in Europe, women over 50 years have a 14% chance of getting a hip fracture. The lifetime risk of osteoporotic fractures is around 30-40% (Kanis 1994). Breast cancer patients have an even higher risk, because, in addition to osteoporosis caused by normal hormonal changes (menopause), cancer treatments (radiation and chemotherapy), adjuvant treatments (e.g. tamoxifen) as well as ovariectomy or ureterectomy also cause osteoporosis (Bruning et al. 1990, Pouilles et al. 1994, Powles et al. 1996, Delmas et al. 1997, Saarto et al. 1997). Hormonal replacement therapy could prevent the risk for osteoporosis. However, it is not used in breast cancer patients as much as in healthy women because it is believed to facilitate cancer cell growth (Bergkvist et al. 1989).

The proportion of malignant cells in a solid tumor that reach the circulation is small, and an even smaller proportion cause metastases. In order for metastases to develop, individual tumor cells need to separate from surrounding cells, penetrate either blood vessels or lymphatics, make contact with tissue, and then grow and divide. Angiogenesis must also be initiated. In cancer patients, the normal bone loss caused by aging and cancer treatments such as cytostatics and radiation weakens the bone, which makes it more vulnerable to the tumor cells and formation of metastases (Healey 1997). Additionally, local or general osteolysis contributes to the appearance of metastases, since tumor cells release transmitter agents that activate osteoclasts, and this creates a favorable environment for metastases (Stewart et al. 1980, Fleisch 1991). Bone metastases are known to cause severe pain (Healey 1997). Bone is one of the most common target tissues for metastases in breast cancer (American Cancer Society 2013). Bone metastases are more resistant to chemotherapy than soft tissue metastases, and after detecting bone metastases the survival prognosis for breast cancer patients is normally only a couple of years (American Cancer Society 2013). Therefore, it is important to protect bones and make them more resistant to cancer cell invasion.

2.1.2 Association of periodontitis, dentin hypersensitivity and osteoporosis

The same processes that lead to loss of bone in the spine and hips can also lead to loss of the alveolar bone of the jaws, resulting in periodontal disease, loose teeth, and tooth loss. Gingivitis is a reversible inflammatory response to bacterial plaque buildup that is limited to the gingiva. If unchecked, gingivitis progresses to

periodontitis, an inflammation of the supporting tissues of the teeth including the gingiva, alveolar bone, and periodontal ligament. Periodontitis leads to progressive and irreversible loss of bone and periodontal ligament attachment, as inflammation extends from the gingiva into adjacent bone and ligament (Buencamino et al. 2009).

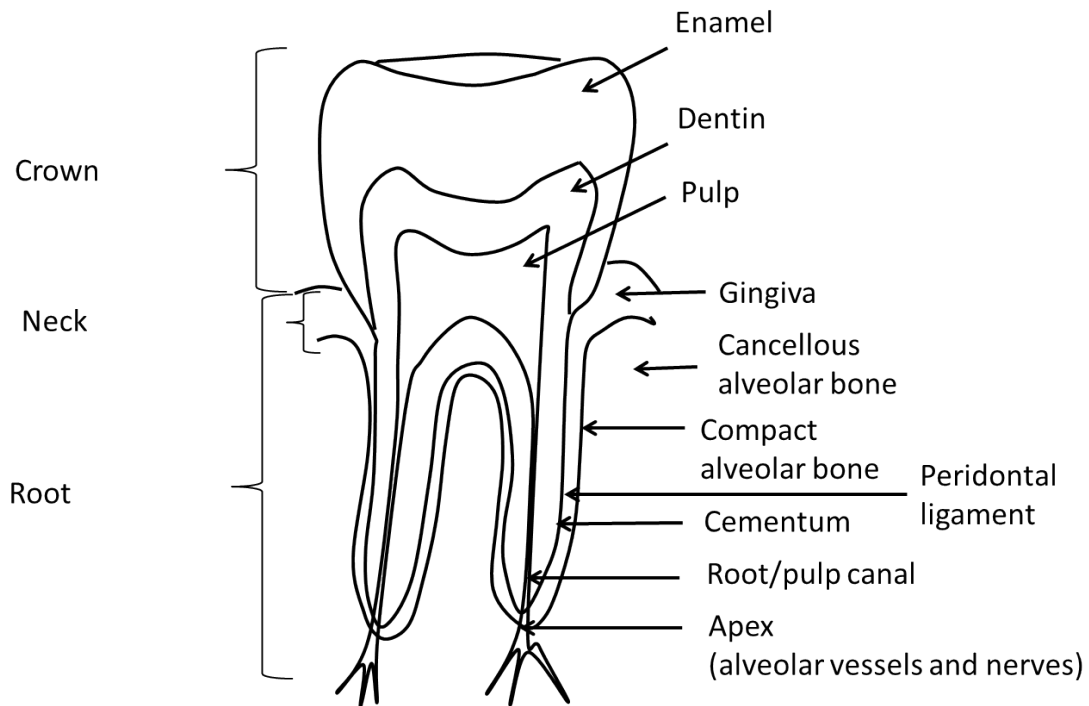


Figure 4. *The anatomy of tooth.*

Dentine forms a hard tissue frame of a tooth surrounding a sensitive pulp, which is rich in nerves and blood vessels (Fig. 4). Dentine is covered by enamel on the crown and cementum on the root. These layers protect the dentin zone and prevent its exposure to irritation from the oral cavity. Dentine contains 30 000 – 40 000 dentinal tubules per mm², each being 1-2 μ m in diameter. They extend from the pulp to the enamel or cementum-dentine border. If dentine is exposed, open dentinal tubules form a link between the tooth surface and the pulp. Dentinal tubules contain tissue fluid, and a strong capillary force prevails in the tubules. If fluid is removed, for example mechanically with a dental instrument, blowing air to this area or penetration by solutions with strong osmotic pressure (e.g. sweet solutions) causes an outward flow of fluids from the pulp in the tubule. This flow of fluids is sensed via pulp nerves as a strong pain and such teeth are considered hypersensitive.

Periodontitis is considered to start as plaque induced gingivitis, a reversible condition that, if left untreated, may develop into chronic periodontitis (Fig. 5). Periodontitis is an inflammation of the supporting tissues of the teeth. It leads to a

progressive loss of alveolar bone and periodontal ligament attachment as inflammation extends from the gingiva into deeper periodontal structures. In periodontal disease the height of alveolar bone is lower which results in deeper pockets (Fig. 5). The clinical symptoms of periodontitis include color, texture and volume alterations of the marginal gingiva, bleeding on probing (BOP) from the pocket area, increased pocket depth, loss of probing attachment level, recession of the gingival margin, loss of alveolar bone, root furcation exposure, increased tooth mobility, drifting and, eventually, tooth exfoliation (Buencamino et al. 2009).

The aim of periodontal therapy is to reduce gingival inflammation, and, in the case of periodontitis reduce pocket depth as well as bone loss and increase attachment to gingiva. Usually this goal can be accomplished by non-surgical means in patients with gingivitis or moderate periodontitis, whereas severe cases, particularly those involving intrabony defects, also require periodontal surgery. The objective of periodontal surgery is to provide access for proper instrumentation and cleaning of the root surface. In addition, elimination or reduction of soft tissue in the periodontal pocket may be needed. Moreover, lost periodontal support will also be restored if possible. Periodontal treatment, both surgical and non-surgical, results in recession of the gingival margin after healing. Localized recession and root exposure is often associated with dentin hypersensitivity (Isidor et al. 1984a, 1984b, Gillam et al. 2002, Buencamino et al. 2009).

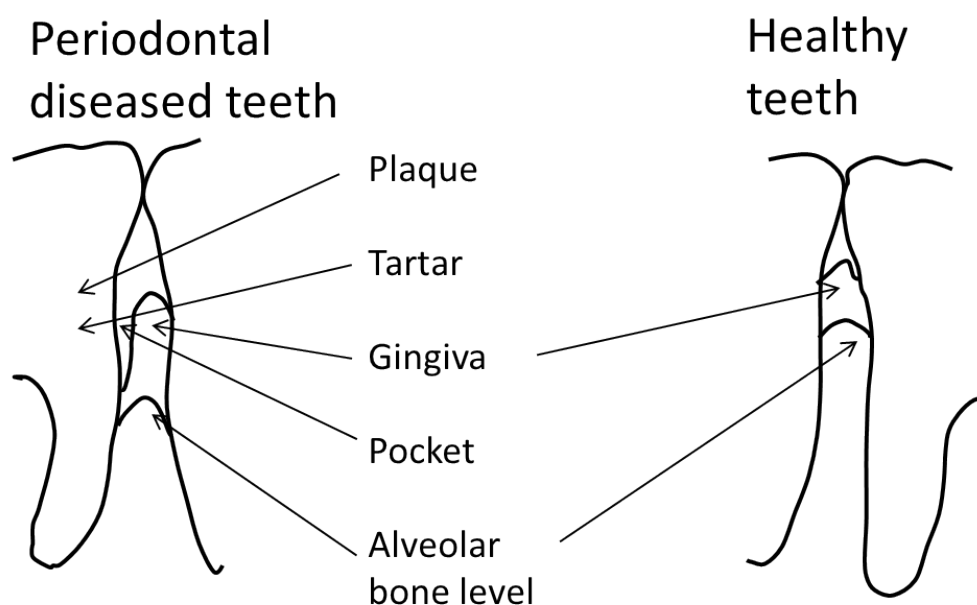


Figure 5. Healthy gingiva, soft tissue attachment and alveolar bone level (right) vs periodontal diseased teeth with plaque, tartar, deeper pocket and reduced alveolar bone level (left).

Osteoporosis and periodontitis are chronic diseases with an increased prevalence in aging populations. Both diseases involve osteoclastic bone resorption. Local production of cytokines appears to enhance osteoclast-mediated resorption of bone in estrogen deficient patients. Peripheral blood monocytes from patients with osteoporosis secrete more interleukin-1 (IL-1). IL-6 is the most important cytokine in the recruitment of osteoclasts in abnormal osteoporotic bone remodeling such as in postmenopausal women (Giuliani et al. 1998). The process of periodontal disease involves mast cells, neutrophils, macrophages, lymphocytes and plasma cells. Macrophages play an important role through the secretion of interleukins 1, 6, 8, 10 and TNF- α . More importantly, osteoporosis and periodontitis have these cytokines in common (Reinhart et al. 1999). Osteoporosis has been recognized as a potential risk factor in the development of periodontitis (Krejci and Bissada 2000). Skeletal bone mineral density appears to be related to interproximal alveolar bone loss and to periodontal attachment loss (von Wöern et al. 1994, Tezal et al. 2000).

2.1.3 Measurement of bone metabolism

Bone strength depends on three factors: density, structure and content, i.e., bone quality. During aging all these three factors change to some extent (Kanis et al. 1994, Kröger et al. 1995). Bone turnover can be analyzed with physical and chemical measurements and histomorphological analyses. In order to detect osteoporosis, bone mineral density (BMD) is measured and compared with that of average BMD for the same age and gender. More detailed information about bone structure and quality requires anamnesis, X-ray examination, laboratory measurements (i.e., chemical measurements) and biopsy (histomorphometric analyses of bone).

In practice, the treatment of bone loss involves the prevention of bone resorption. Thus, the effect of treatment is most easily measured using chemical and physical analysis. The great benefit with chemical measurements is that treatment effectiveness can be measured within one to three months after treatment initiation. In contrast, bone mineral density may only show results after one to two years (Kanis et al. 1994, Kröger et al. 1995).

Bone mineral density is most often determined using dual energy X-ray absorptiometry (DEXA). The measurement is based on dual energy X-ray absorption and densitogram computer analyses. Using dual energy minimizes the potential for errors caused by soft tissue. DEXA indicates the results as surface area densities (g/cm^2). Thus, the effect of bone size on the results is not totally corrected. This means that the determined surface area density would be greater in larger than smaller sized bones even though the real density (g/cm^3) would be the same (Kröger et al. 1992).

With DEXA it is possible to measure central bone (vertebral lumbar spine and upper femur) and total mineral density. The radiation dose is low ($<1 \mu\text{Sv}$; for comparison, the exposure of natural background radiation is 3 mSv every year)

and the repeatability of the results is good (98-99.5%), which makes it possible to follow any treatment effect and change in situation. The accuracy of the measurement is 97% (Faulkner et al. 1993). The most common measurement is lumbar spine anterior-posterior (L2-L4) analysis, which takes about two to five minutes. Upper femur BMD measurement also gives information also about the geometry of the femur. In addition, DEXA measurement can be performed on the peripheral bone area with good repeatability (Faulkner et al. 1993). Fan-beam DEXA measurement is quicker than traditional DEXA and it can be used to determine dimensions of vertebra (morphometry) and thus possible deformities. However, the radiation dose is greater (lumbar spine and upper femour 5-50 μ Sv, total spine 1200 μ Sv) (Kormano 1998). Other methods for bone density measurement are single photon absorptiometry (SPA), single X-ray absorptiometry (SXA), dual-photon absorptiometry (DPA), quantitative computed tomography (QCT) and ultrasound measurements (bone ultrasound attenuation (BUA) and ultrasound tissue visualization (UTV)). Additionally, some guiding assessment of bone density can be obtained using magnetic resonance imaging (MRI) (Kormano 1998). Meta-analysis has shown that bone density measurement can predict bone fractures. One standard deviation reduction in bone density increases the risk for fractures by 1.5 times (95% confidence interval 1.4 to 1.6). In the hip and spine areas the predictability is even better. This means that bone mineral density measurements can be used to identify those who are at greater risk of suffering fractures (Marshall et al. 1996).

The activity of cells involved in bone turnover can be assessed using body fluids and chemical biomarker measurements. The biomarker needs to clearly reflect the variable of interest e.g., bone resorption, and be specific and sensitive enough. Chemical biomarker measurements do not distinguish osteoporotic from “healthy” people, but can be used to determine if the used treatment is effective. It is crucial to understand bone formation in detail in order to be able to make any conclusions based on chemical biomarkers (e.g. bone turnover is higher in children than adults, in postmenopausal than premenopausal women and is greater for several months after fracture during healing). There are several biomarkers that describe bone turnover and they can be divided into those indicating bone formation or bone resorption. Some chemical biomarkers are listed in Table 2.

Table 2. *Biomarkers of bone turnover.*

Bone resorption biomarkers	
Serum	<p>Type I collagen aminoterminal telopeptide (Nt_x)</p> <p>Type I collagen carboxyterminal telopeptide (ICTP)</p> <p>Type I collagen carboxyterminal telopeptide (CrossLaps)</p>
Plasma	Tartrate-resistant phosphatase
Urine	<p>Hydroxyproline</p> <p>Free pyridinoline and deoxypyridinoline</p> <p>Total pyridinoline and deoxypyridinoline</p> <p>Type I collagen carboksyterminal telopeptide (CrossLaps)</p> <p>Type I collagen aminoterminal telopeptide (Ntx)</p>
Bone formation biomarkers	
Serum	<p>Alkaline phosphatase</p> <p>Bone-derived alkaline phosphatase</p> <p>Osteocalcin</p> <p>Osteoprotegerin</p> <p>Type I procollagen</p> <ul style="list-style-type: none"> • Carboxyterminal (PICP) propeptide • Aminoterminal (PINP) propeptide

The biomarker can be involved in a metabolism process as such or alternatively part of the metabolism product. As an example of agent involved is osteocalcin. 1.25-(OH)₂-D-vitamin stimulates osteoblasts to produce osteocalcin. Osteocalcin in serum mainly originates directly from osteoblasts (Garnero et al. 1994). Hydroxyproline, on the other hand, is example of a metabolism byproduct. It is an amino acid, which is typically found in collagen and is released into the blood and then urine as collagen is decomposed (Risteli and Risteli 1993).

The treatment effect (e.g. the effect of clodronate on bone) can be defined using a histomorphometric measurement. Using this measurement, the amount of osteoclasts and area of resorption can be calculated as well as the ratio to osteoblasts and osteoclasts. Histological analysis can also be used to detect possible micrometastases (Elte et al. 1986). However, histological analysis requires an invasive biopsy to be taken. This, combined with laborious histological analysis, prevents histomorphometric measurement from being used as a routine diagnostic tool.

2.2 Clodronate and other bisphosphonates

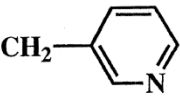
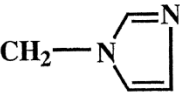
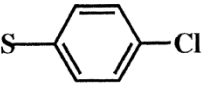
Clodronate belongs to bisphosphonates (BPs) that are pyrophosphate analogs and have a P-C-P backbone. The P-O-P moiety of pyrophosphate is replaced by a P-C-P moiety to make the BPs resistant to enzymatic hydrolysis. Naturally occurring pyrophosphate regulates mineralization of the bone matrix (Fleisch 1991, Plosker and Goa 1994). The basic P-C-P structure of BPs allows many possible variations, by changing the two lateral chains on the carbon atom. Some common bisphosphonates are: etidronate, clodronate, tiludronate, pamidronate, alendronate, risedronate, zoledronate and ibandronate. The most common bisphosphonates used clinically are presented in Table 3 (Ezra and Golomb 2000). Their antiresorptive potency varies (per weight). If etidronate is defined as having a potency of 1, then for example clodronate has a potency of 10, tiludronate 10, pamidronate 100, alendronate 100-1000, risedronate 1000-10 000 and zoledronate more than 10 000. The oral bioavailability also differs among bisphosphonates (e.g. pamidronate 0.3%, risedronate 0.65%, alendronate 0.75%, clodronate 1-2%, etidronate 3-7%, tiludronate 6%) (Watts 1998).

Like all BPs, clodronate is highly negatively charged at the intestinal physiological pH. Clodronate has four pK_a values, depending on the side chain structure: 1.7, 2.1, 5.7 and 8.3 (Fonong et al. 1983). It has been demonstrated that bisphosphonates are only active when at least three ionisable groups are presented (Van Gelder et al. 1995). Clodronate is highly water-soluble (Pentikäinen et al. 1989).

Bisphosphonates inhibit systemic bone resorption, prevent osteoclast development and induce or cause osteoclast apoptosis (Rogers et al. 2000, Green 2003). The mechanism of action is reduction of osteoclastic activity (Sato and Grasser 1990). Clodronate prevents bone loss by binding to active sites of bone

remodeling, inhibiting osteoclast mediated bone resorption, preventing osteoclast development from hematopoietic precursors (Hughes et al. 1989) and production of an osteoclast inhibitory factor (Hughes et al. 1995). The affinity of bisphosphonates to bone mineral hydroxyapatite is the basis for their use as inhibitors of ectopic calcification and of bone resorption.

Table 3. *Most common bisphosphonates in clinical use.*

$ \begin{array}{c} \text{OH} \quad \text{R}_1 \quad \text{OH} \\ \quad \quad \\ \text{O}=\text{P}-\text{C}-\text{P}=\text{O} \\ \quad \quad \\ \text{OH} \quad \text{R}_2 \quad \text{OH} \end{array} $		
<u>Name</u>	<u>R₁</u>	<u>R₂</u>
Etidronate	OH	CH ₃
Clodronate	Cl	Cl
Pamidronate	OH	CH ₂ CH ₂ NH ₂
Alendronate	OH	CH ₂ CH ₂ CH ₂ NH ₂
Ibandronate	OH	$ (\text{CH}_2)_7\text{N} \begin{cases} \text{CH}_3 \\ (\text{CH}_2)_4-\text{CH}_3 \end{cases} $
Risedronate	OH	
Zolendronate	OH	
Tiludronate	H	

The physicochemical effect of clodronate includes binding strongly to the CaP crystals and inhibiting their growth, aggregation and dissolution (Francis 1969, Francis et al. 1969, Fleisch et al. 1970, Meyer et al. 1973, Hansen et al. 1976). *In vivo* action is however mediated through mechanisms other than the

physicochemical inhibition of crystal dissolution. Clodronate affects osteoclasts both directly and indirectly. The direct interaction with osteoclasts and/or osteoblasts involves a variety of biochemical pathways (*e.g.*, receptor activator of NF-KappaB, transforming growth factor and bone morphogenic proteins mediated pathways) (Fleisch 1997, Kirkwood et al. 2007). Osteoclast activity is inhibited as the osteoclast engulfs clodronate containing bone (Fleisch 1997). Clodronate also directly induces tumor cell and osteoclast apoptosis and reduces cell adhesion and invasion in pre-clinical models (Hughes et al. 1995, Fromigue et al. 2003, Santini et al. 2003). Additionally, BPs have indirect effects via osteoblasts. *In vitro* evidence suggests that BPs inhibit osteoclast recruitment and survival by acting on cells of osteoblast lineage (Sahni et al. 1993). BPs reduce osteoblasts ability to produce osteoclast stimulating agent and more importantly stimulate osteoblast activity to secrete an inhibitor for osteoclast recruitment and survival (Sahni et al. 1993, Fleisch 1997).

2.2.1 Pharmacokinetics and bioavailability

The oral bioavailability of bisphosphonates is poor, due to high ionization at physiological pH making them very polar and poorly absorbed from GI tract. In addition, the BPs have been associated with adverse GI effects in humans. There is also great within- and between-subject variability in absorption. Within the same subject, the amount absorbed from a single oral dose of clodronate calculated by AUC can vary up to 8-fold (Castren-Kortekangas et al. 1997). From subject to subject, 30-fold differences in the AUC are possible, and the standard deviation is usually of the same magnitude as the mean AUC value (Saha et al. 1994). The absolute bioavailability of clodronate from two different oral doses (clodronate 800 mg or 1600 mg as 400 mg per capsule) was 1.9% for a smaller dose and 2.1% for a 1600 mg dose. The difference in the absolute bioavailability of these two doses was statistically not significant. Both treatments were well tolerated, and the adverse event profiles were similar in the different treatment groups (Villikka et al. 2002). Additionally, two different clodronate preparations: an 800 mg tablet and a 400 mg capsule were studied to compare bioavailability. Assuming 20% binding to bone and 80% excretion of the absorbed clodronate, the gastrointestinal absorption calculated on the basis of the pooled urinary excretion data were identical (geometric mean 2.4–2.5%) for the two preparations (Castren-Kortekangas et al. 1997).

The half-life of circulating BPs is short, in rats it is in the order of minutes, in humans it is somewhat longer. The peak serum concentration after a single oral dose of clodronate is reached in half an hour, which may point to site-specific absorption in the (upper) GI tract. Absorption occurs to some extent in the stomach and to a larger extent in the small intestine. It is greatly diminished when the drug is given with meals, especially in the presence of calcium and iron (Fleisch 1991, Plosker and Goa 1994). Elimination of clodronate from serum is characterized by two clearly distinguished phases: the distribution phase with a

half-life of about two hours (Conrad and Lee 1981, Yakatan et al. 1982) and a very slow second elimination phase (Yakatan et al 1982, Pentikäinen et al. 1989, Saha et al. 1994), which results from its strong binding to bone (Pentikäinen et al 1989, Saha et al. 1994). Therefore, the rate of entry into bone is very fast.

The values of skeletal uptake in humans are about 20% for clodronate, 50% for etidronate and more for pamidronate and alendronate (Fleisch 1997). When BPs are given in clinically effective doses, there seems to be no saturation in their total skeletal uptake in humans (Fleisch 1991, Plosker and Goa 1994).

2.2.2 Clinical use

Clodronate has been shown to prevent or delay skeletal-related events and decrease bone pain as well as normalize calcium levels in the presence of hypercalcemia. Clodronate has been used clinically for: hypercalcemia and osteolysis due to malignancy, postmenopausal women with vertebral compression fractures, postmenopausal women with total hip bone density T-score below -2.5 (osteoporosis), elderly men with non-traumatic fractures, some patients with secondary osteoporosis due to corticosteroids, Paget's disease, cancer metastatic to bone, and other bone diseases with high bone resorption. It can also relieve pain and improve patient functioning and quality of life (Plosker and Goa 1994).

In general, clodronate is a well-tolerated therapy. However, it has also disadvantages, which are mostly associated to the oral, systemic administration. The most common adverse effect is GI disorder, reported in 2-10% of the patients (Hannuniemi et al. 1991, Mian et al. 1991, Diel et al. 2007). Other frequently reported adverse events such as decreased serum calcium, signs and symptoms of hypocalcaemia, renal failure and pain at the injection site are associated with parenteral administration (Plosker and Goa 1994). Additionally, there are concerns about bisphosphonate-associated osteonecrosis of jaw, which, although a rare disorder, is also associated with systemic oral or intravenous administration. It is characterized by exposure and loss of bone in the maxillofacial complex that is resistant or refractory to conventional therapy (Carey and Palomo 2008). This information is mostly based on case reports involving cancer patients treated with high intravenous doses and who had other risk factors for jaw disease (Ruggiero et al. 2004, Woo et al. 2006, Palomo et al. 2007, Dodson et al. 2008). The American Dental Association released a statement noting that osteonecrosis of the jaw can occur with or without bisphosphonate use (The American Dental Association 2006). The incidence of jaw necrosis in patients receiving bisphosphonate therapy for the treatment of osteoporosis appears to be significantly lower compared to that with intravenous continuous therapy and seems to be mostly related to nitrogen containing bisphosphonates (Ruggiero et al. 2004, Ruggiero et al. 2009). For example, as less than 1% of an oral dose of an aminobisphosphate, pamidronate, is absorbed from the GI tract (Ezra and Golomb 2000), and more than 50% of the dose given intravenously is bioavailable (Berenson et al. 1997), the possibility of osteonecrosis of the jaw is unlikely with oral administration.

There are cases where rash (Pajus et al. 1993) and bronchoconstriction in an aspirin-sensitive asthmatic patient (Rolla et al. 1994) have been reported but also in these cases clodronate was administered intravenously.

2.2.3 Bisphosphonates in the treatment of periodontal disease

Even though BPs are mostly used to treat skeletal-related bone events like those described above, recent studies suggest that bisphosphonates slow the resorption of alveolar bone of the maxilla and mandible as well and have been noted to improve periodontal status (El-Shannawi and El-Tantawy 2003, Rocha et al. 2004, Palomo et al. 2005, Jeffcoat et al. 2007). In all these studies BPs were given orally. For example Palomo et al. (2005) showed that women with diagnosed osteoporosis using risedronate for three months had significantly less plaque accumulation, less gingival inflammation, lower probing depths, less periodontal attachment loss, and greater alveolar bone levels. In the study conducted by Jeffcoat et al. (2007), risedronate was given for two years. In the subgroup of patients with low mandibular BMD at baseline, alendronate significantly reduced bone loss compared to placebo. However, that effect was not seen in patients with normal baseline mandibular BMD.

The possibility of using bisphosphonates for the management of periodontitis has been studied in animals. Alendronate was shown to inhibit bone loss around affected teeth in comparison to controls (Brunsvold et al. 1992, Reddy et al. 1995, Chavassieux et al. 1997). Local use of risedronate has been shown in rats to stimulate alveolar bone growth (Adachi et al. 1994, Binderman et al. 2000, Yaffe et al. 2000) and to increase bone formation around dental implants when used in peri-implant defect regeneration (Igarashi et al. 1996). Although in human studies BPs were given systemically and in all these animal studies the treatment was given locally, surgically or injected, the results are encouraging for the potential management of periodontal disease in a noninvasive local manner.

2.3 Bioactive glass

Bioactive glass ($\text{Na}_2\text{O}-\text{CaO}-\text{P}_2\text{O}_5-\text{SiO}_2$) is known to induce or aid osteogenesis in physiological systems (Hench and Paschall 1974) and would appear to be a suitable material for surface reactivity. There are several bioactive glasses with slightly different compositions. The first bioactive glass investigated is known as Hench glass (BG, 45S5) having the composition of SiO_2 45%, Na_2O 24.5%, CaO 24.4% and P_2O_5 6% (weight percentages). It is considered as the model of all bioactive glass compositions. The bioactive glass used in this study contains SiO_2 53%, Na_2O 23%, CaO 20% and P_2O_5 4% (weight percentages), and is known as S53P4 (BAG). This glass was chosen as it is the bioactive glass produced in Finland and was thus readily available. Additionally, it is widely investigated and clinically used. It is amorphous and soluble in water, has a density of 2.66 g/cm^3 ,

is odorless and is available commercially as white granules with the following particle size ranges: 0.5-0.8 mm, 0.8-1.0 mm, 1.0-2.0 mm and 2.0-3.15 mm.

In the technical sense, an inorganic glass is typically a product of fusion which has been cooled to a rigid condition without crystallizing (Hench and Wilson 1999). Many inorganic glasses contain silica as their main component and glass former. Like all glasses, bioactive glasses contain other molecular constituents within the amorphous form. In the original form currently approved for medical use, the glass is a four-component system of oxides of silicon, calcium, sodium and phosphorus. The relatively low silicon and high alkaline content lead to a rapid ion exchange in aqueous environments. This exchange generally leads to an increase in solution pH, which can be substantial for finely grained powders having high surface to volume ratios (Hench and Wilson 1999, Felipe et al. 2009). Therefore, it is understandable that the activity of bioactive granules strongly depends on particle size, with increasing activity as particle size decreases (Felipe et al. 2009).

When BAG is implanted into a bone defect, the surface of the BAG remodels to form hydroxy-carbono-apatite (HCA) or as it is commonly called hydroxyapatite (HA), the chemical and structural equivalent of bone mineral (Hench and Wilson 1999). Additionally, bone growth markers and bone repair cells are increased in the presence of bioactive glass, thus accelerating for example the overall healing process of bone. The initially rapid release of sodium is accompanied by a somewhat slower release of other ion species, predominantly calcium and silica. Under certain conditions in solution precipitates to form calcium-containing mineral layers occurs onto the glass and onto other nearby surfaces. In this case, the outer glass surface itself can transform to HA. The ability to build such a surface is sometimes referred to as a measure of the “bioactivity” of the glass. When implanted into the body, repair cells will colonize the bioactive surface, laying down new tissue on and into the glass.

The calcium phosphate formation at the surface of bioactive glass *in vitro* proceeds in stages (Fig. 6) (Lockyer et al. 1995, Hench and Wilson 1999). When immersing the glass in tris(hydroxymethyl)aminomethane (TRIS) solution or in simulated body fluid (SBF) a CaP-rich surface layer forms. This accumulation takes place within the silica structure. First there is an ion exchange. Later, apatite crystals forming spherulites appear on the surface. The Ca/P-ratio of initially formed CaP is about unity. It is proposed that this is due to bonding of phosphate to silica gel. The surface is stabilized, *i.e.*, leaching is delayed, by the rapid Ca and P-accumulation within the silica structure before apatite crystals are observed on the surface. It is proposed that the initially formed CaP is initiated within the silica gel. The crystallizing surface of apatite provides nucleation sites for extensive apatite formation on the glass surface. In the presence of citrate, no Ca and P-accumulation occurs at the glass surface, and instead soluble Ca-citrate complexes form (Andersson and Kangasniemi 1991, Hench and Wilson 1999). The formation of a CaP layer was found to happen and continue only when the solution was continuously replenished and only if this solution contained electrolytes and proteins (Andersson and Kangasniemi 1991). Without solution replenishment, the

solution quickly became saturated in silicon, and the silicon no longer dissolved. When the glass was immersed in a solution with serum, a porous surface structure with fine precipitate formed, in contrast to a dense surface reaction layer with closely packed globular precipitates that was formed in a solution without serum. The combined effect of continuous solution replenishment and the use of a solution containing serum proteins led to the formation of a surface reaction layer that did not prevent continuation of the process. As such, all silicon (Si) was released, and eventually a hollow CaP shell was formed. Thus this supports the hypothesis that there is a physico-chemical mechanism of Si transport through the CaP-rich layer followed by Si dissolution.

Reaction stages of a bioactive glass can be divided to five different stages (first five stages in Fig. 6) (Lockyer et al. 1995, Hench and Wilson 1999). Firstly, sodium ions are replaced by hydrogen ions ($\text{Si-O-Na}^+ + \text{H}^+ + \text{OH}^- \rightarrow \text{Si-OH} + \text{Na}^+ + \text{OH}^-$) to form silicate. After that silicate breaks up to form silanols. As a result of breaking Si-O-Si bonds, there is loss of soluble silica in the form of $\text{Si}(\text{OH})_4$ into solution and formation of silanols, Si-OH ($\text{Si-O-Si} + \text{H}_2\text{O} \rightarrow 2\text{Si-OH}$). At the third stage, silanols undergo equilibrium condensation and partial repolymerization to form a SiO_2 -rich gel layer. After that, in the following stage, the reaction continues with formation of an amorphous CaP phosphate layer by rapid migration of calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions through the SiO_2 -rich layer to the surface with the $\text{CaO-P}_2\text{O}_5$ -rich film forming on the top of the SiO_2 -rich layer. This amorphous CaP layer becomes thicker and denser with the arrival of more ions from bulk glass and absorption of calcium and phosphate ions from the physiological solutions. In the final stage, the amorphous CaP crystallizes into a mixed hydroxyl-, carbonate- and fluoro-apatite layer (OH^- , CO_3^{2-} and F^- anions being derived from host tissue) (hydroxyapatite = HA = $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ =calcium hydroxyapatite).

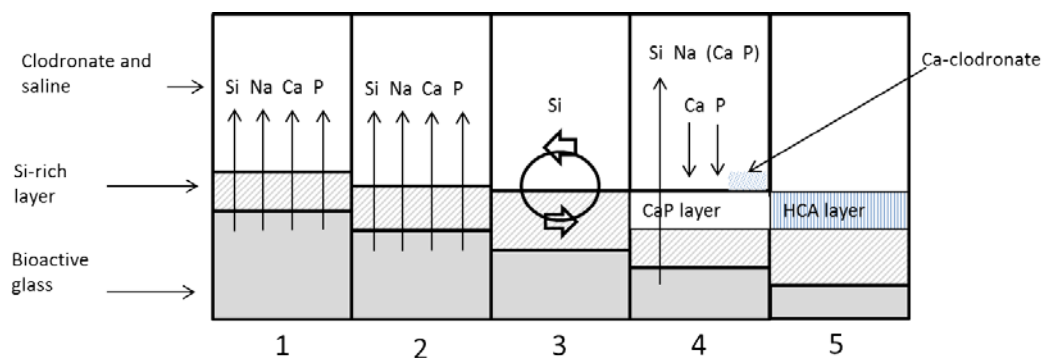


Figure 6. Schematic description of surface reactions, Si-rich, CaP, calcium clodronate (Ca-clod) and Hydroxyapatite (HA) layer formation (first five stages 1-5) and bone bonding (stages 6 and 7) of bioactive glass.

2.3.1 Tissue bonding

Tissue bonding properties of bioactive glass have been described in literature. However, those are not fully proven and need more investigation and evidence. Seven different stages of change have been described on the bone tissue side of the interface with bioactive glass. They involve adsorption of biological moieties in the HA layer, action of macrophages, attachment of osteoblast stem cells, differentiation of stem cells, generation and crystallization of matrix and finally proliferation of bone (Filho et al. 1996). The structure of the interfacial bonding in hard tissue contains different stages, with the first five stages overlapping with the bioactive glass surface reactions (Fig. 6). An amorphous CaP-rich layer bonds the BAG and healing bone together. The formed layer crystallizes into HA agglomerates within seven to ten days in tissue contact and after four days in simulated body fluid. In the presence of collagen fibers produced by osteoblasts, apatite crystallizes and collagen becomes structurally integrated within the apatite agglomerates and vice versa. Mucopolysaccharides significantly enhance the physiochemical interaction between the crystallizing apatite layer and collagen. After formation of the amorphous cementing zone on the CaP-rich layer, the subsequent steps in bone development and bonding are managed by osteoblasts in the contact area. If insufficient surface reactivity is present, osteogenesis does not proceed (Hench and Paschall 1974, Hench and Wilson 1984, Hayakawa et al. 1999). At the bonding interface, osteoblasts provide collagen, ground substances and matrix vesicles for primary mineralization. Later, the whole area is mineralized, the osteocytes become rather evenly distributed (Hench and Wilson 1984).

The bonding between bioactive glass surface and soft tissue is formed by attachment of collagen fibers to a silica rich and HA containing layer (Wilson et al. 1981). Soft tissue bonding is a valuable property for example in oral applications (Hench and Wilson 1984) and there are results reporting tissue attachment to bioactive glass (Andersson et al. 1994, Tuominen et al. 1995). At the surface of the bioactive glass, the amorphous CaP crystallizes and, simultaneously, collagen fibers from fibroblasts or osteoblasts integrate within HA agglomerates (Hench and Paschall 1974, Hench and Andersson 1999).

2.3.2 Clinical use

Bioactive glass has been used clinically ever since the Second World War and has been investigated for several decades with respect to different clinical applications and physicochemical properties (Hench and Wilson 1999). In the cranio-maxillofacial area, BAG can be used as a bone cavity filling material, frontal sinus obliteration after severe chronic sinusitis or fractures in the frontal bone area, mastoid sinus obliteration and nasal cavity narrowing (Stoor et al. 2001, Peltola et al. 2006, Stoor et al. 2010). BAG can be used also in orthopedics as a bone cavity filling material (Lindfors et al. 2009). Recent evidence shows that BAG can be used also to treat osteomyelitis, which is infection in bone caused by several bacteria sometimes resistant to antibiotics and can lead to sclerosis and deformity

if in chronic form (Lindfors et al. 2010). In this study, patients with verified chronic osteomyelitis in the lower extremity or the spine were treated with BAG as a bone substitute with good results. Additionally, bioactive glass has been used in dental applications for the repair of cortical bone defects (Turunen et al. 1998) and in the treatment of hypersensitive teeth (Banerjee et al. 2010, Tirapelli et al. 2011). Based on numerous studies with this material and clinical use, the material can be considered well tolerated and safe to use. Bioactive glass is known to have no cytotoxicity (Kuo-Chun et al. 2007, Zhou et al. 2010).

2.4 Combination of bisphosphonate and bioactive glass

There are no published data available about the use of bioactive glass and clodronate in combination. However, there are a couple of studies which have investigated bone formation using a combination of bioactive glass and other bisphosphonates administered locally in surgery as a filling material for bone defects. Välimäki et al. (2006) studied the effect of zoledronic acid and BAG as a bone graft substitute in rat tibia. Zoledronic acid was administered subcutaneously. They concluded that the beneficial effect of bisphosphonate therapy may extend to healing of implants with bioactive hydroxyapatite coatings. In the other study (Srisubut et al. 2007), the combination of alendronate and bioactive glass were implanted in the rat mandible as a filling material in a surgical created bone defect. The experimental groups were bioactive glass soaked with the alendronate solution or bioactive glass soaked with saline. After four weeks, all animals were sacrificed and the number of osteoclasts and the amount of new bone formation were evaluated. Based on histologic results, the combination was found to induce more bone regeneration and was considered to be useful for alveolar ridge augmentation followed by dental implant surgery and for bone regeneration in periodontal defects. No difference was found when the numbers of osteoclasts were compared.

The affinity of bisphosphonates to bone mineral hydroxyapatite is known (Fleisch et al. 1970) and as bioactive glass remodels to form HA, the chemical and structural equivalent of bone mineral, it could be assumed that clodronate and BAG could form a complex that would benefit both. This novel combination enhances the bioactivity of BAG. Simultaneously, clodronate can contribute to the attachment to both soft tissue and bone. Together BAG and clodronate form a totally new approach for local delivery.

3 Aims of the study

The aim of this study was to investigate the effects of clodronate, given either orally or locally, to the quality of bone, and to develop a combination product including clodronate and bioactive glass for local administration for periodontal application. Additionally, the adverse effects of clodronate in oral administration and effects of combination product for surrounding tissue in local administration were evaluated.

More specifically, the aims of this thesis were:

1. To study the effects of oral adjuvant clodronate (systemic administration) on bone quality by evaluating the BMD and incidence of bone metastases, other metastases, and survival in patients having surgery, radiotherapy, and/or drug treatment for primary operable breast cancer (**I, II**).
2. To develop and characterize a combination product of clodronate and BAG for the local administration to be used in periodontal application (**III, IV**).
3. To investigate the effect of this novel combination product administered locally in the treatment of periodontitis maintenance phase (**V**).

4 Experimental

4.1 Materials

4.1.1 Breast cancer patients and material (I, II)

The multicentre, double-blind trial included more than 1000 patients (1069) with histologically or cytologically confirmed primary operable breast cancer. These patients were randomized to receive orally either four capsules of clodronate (Bonefos®, Leiras OY, Helsinki, Finland; 530 patients) or four capsules of an identical placebo (539 patients) for two years. Capsules were to be taken at least half an hour before or after eating.

Patients were treated for the breast cancer with surgery, radiotherapy, systemic chemotherapy and/or endocrine therapy. The trial centers treating patients were the Royal Marsden Hospital, London and Sutton, United Kingdom; the Tom Baker Cancer Centre, Calgary, Canada; 20 hospitals in Norway; The Hôpital St Luc, Montreal, Canada and the Central Hospital, Jyväskylä, Finland. The BMD analyses and reviews were done in the University of Sheffield, World Health Organization Metabolic Bone Unit, Sheffield, United Kingdom. The BMD data were collected and analyzed for patients in the Royal Marsden Hospital, Sutton and the Tom Baker Cancer Centre (I). The BMD data included data from 311 patients (156 active and 155 in placebo group).

4.1.2 Bioactive glass, clodronate and hydroxyapatite (III-V)

Bioactive glass (S53P4), BonAlive™ is a CE-marked (medical device, with marketing authorization in European Union) product by BonAlive Biomaterials Ltd, Finland (former Vivoxid Ltd). BonAlive™ contains four oxides (SiO₂ 53%, Na₂O 23%, CaO 20% and P₂O₅ 4% w/w). It consists of amorphous odorless white granules and the particle size ranges of: 0.5-0.8 mm (III-V) and <0.5 mm (IV) were used, which had a density of 2.66 g/cm³. The bioactive glass was used either as dry granules or 1 g of the product was moisturized with 900 µl of 0.9% saline. Clodronate was developed by PharmaZell GmbH (Lot No T07/009 Ph. Eur.). It was in the form of disodium salt (CH₂Cl₂O₆P₂Na₂ 4H₂O 360.9 g/mole). A combination of 1 g of bioactive glass and 100 mg (IV), 200 mg (III-V) or 300 mg (IV) clodronate (corresponding to the base) was used as dry powder and with 900 µl (III-V) or 1350 µl (IV) of 0.9% saline. Hydroxyapatite (calcium phosphate tribasic HCa₅O₁₃P₃), Sigma Aldrich Co., St.Louis, USA was used as a reference (III, IV) and calcium hydroxide (H₂CaO₂) Sigma Aldrich Co., St.Louis, USA was used to form calcium clodronate (CaClod.) (III).

4.1.3 Periodontal patients and material (V)

An open single-centre, comparative clinical investigation was undertaken with 10 subjects who had periodontitis (at least two periodontal pockets with 4-6 mm pocket depth) and had received primary mechanical anti-infective treatment for the disease. The subjects had two treatments (one per diseased pocket *i.e.*, two pockets treated) each and acted as their own comparator or control. One residual pocket was subgingivally treated with the test product containing BAG and clodronate, and the other one was administered with the comparative BAG product.

The study included four clinical adjuvant treatment application visits once a week and two follow-up visits (one week and five weeks after the last product application). Thus, the duration of the study was about two months for a single participant. During treatment application visits, teeth were treated with studied products for 10 minutes. Treatments were given at the Institute of Dentistry, Faculty of Medicine, University of Turku.

The test product included a combination of 1 g BAG particle size 0.5-0.8 mm (S53P4), BonAlive™ and 200 mg of clodronate (see 4.1.2) or BAG alone diluted to 900 µl of 0.9% saline.

4.2 Methods

4.2.1 Data collection and analyses in clinical studies (I, II, V)

In the first two publications (**I**, **II**) pre-randomization assessment included clinical history and physical examination, hematologic, renal, and hepatic blood tests, urinary calcium, hydroxyproline and creatinine, and skeletal radiographs. Computed tomography scanning, magnetic resonance imaging, and bone scintigraphy were undertaken as clinically indicated. In a subset of patients bone mineral density was measured by dual energy X-ray absorption (DEXA, Hologic QDR 1000 densitometer, Vertec Scientific LTD, Reading, UK) (**I**). Analyses for BMD were based on the percent change in BMD from baseline in the spine and hip (**I**). Radiologic assessments for bone metastases were repeated at 24 and 60 months and also when clinically indicated. Hematologic and biochemical tests were repeated at 3-month intervals for the first year, 6-month intervals until 5 years, and then annually. Side effects, adverse events, and compliance were evaluated at each visit. The study had favorable opinion from the ethics committees and was approved by national health authorities. All study centres were regularly monitored on site and case report forms verified in accordance with standards for Good Clinical Practice. Analyses were based on intent-to-treat according to a predetermined analysis plan. Analyses and figures were made using SAS software (version 6.12 for Windows; SAS Institute, Cary, NC). All survival analyses were performed using Cox proportional hazards model (**I**, **II**).

In the study including periodontitis subjects (**V**), baseline measures were obtained before the first adjuvant treatment application during the first treatment visit after screening. This included gingival crevicular fluid (GCF) sampling, and clinical measurements from the two selected tooth sites. The study included a total of four clinical adjuvant treatment visits (Fig. 7) (**V**).

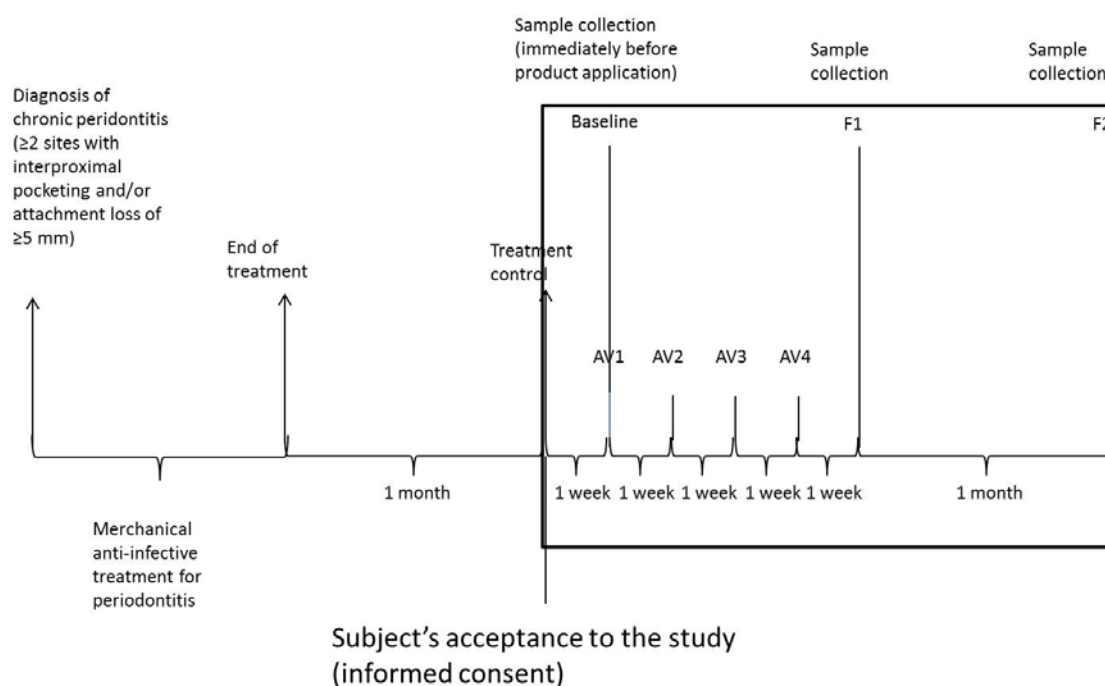


Figure 7. The flow chart presenting the study protocol (**V**) (AV1-4 adjuvant treatment visits 1-4; F1-2 follow-up visits 1 and 2).

At the enrolment visit, the following demographic data were recorded: age, gender, and ethnic origin. Information on relevant previous and current diseases (medical history) was documented. Throughout the investigation, periodontal status at the tested sites was recorded, and was comprised of the following clinical parameters: visible plaque index (VPI, a four-point scale based on the visible continuous plaque along the gingival margin after staining) and bleeding on probing (BOP) dichotomously (present/absent), probing pocket depth (PPD, the distance between the gingival margin and the bottom of the pocket), gingival recession (the distance between the cemento-enamel junction and the gingival margin) and probing attachment level (PAL; derived from PPD and gingival recession) in millimeters measured with a WHO probe. Additionally, overall satisfaction (better, unchanged, worse) since last visit was evaluated at each visit starting at second visit.

Other variables for the study were assessment of pain and study subject's subjective evaluation of the situation. Pain was recorded after cold water and air blow using a visual analog pain scale (VAS; 0=no pain, 10=worst pain). The VAS method includes a visual line of ten centimeters. Study subject is asked to mark the point that best describes the amount of pain she or he felt. Patients were also asked to evaluate their situation compared to the situation at earlier visit. This evaluation was done at every visit starting from second visit with scale: excellent (1), good (2), satisfactory (3), poor (4).

Adverse events and device effects were collected starting from the screening and throughout the investigation. Additionally, all concomitant medications and treatments, together with their purpose for administration, were recorded during all visits.

During the study gingival crevicular fluid (GCF) samples were collected for biomarker analysis indicating bone metabolism. GCF samples were collected three times, at baseline and during the two follow-up visits. After removing supragingival plaque and avoiding any saliva contamination, GCF sampling was performed by placing a paper strip into the selected residual pocket for 30 s. Any paper strip containing blood was discarded. The samples were placed into the Eppendorf tubes, which were stored at -20°C until assayed. The GCF samples were analyzed for two bone remodeling biomarkers, osteoprotegerin (OPG) and osteocalcin, by using a commercially available kit (Milliplex HBN1A-51K Osteoprotegerin/Osteocalcin, Merck Millipore, St. Charles, Missouri, USA) according to the manufacturer's instructions. Osteoprotegerin (OPG) and osteocalcin are two bone remodeling biomarkers.

Data were collected into case report forms. Due to the nature of this investigation no statistical evaluations were originally intended because of the limited number of subjects which might restrict statistical analysis. The data were presented in terms of descriptive statistics as preliminary evidence. All analyses were exploratory and no statistical hypothesis is stated in the plan. However, some of the data are presented in a quantitative format (*e.g.* mean or median) and statistical analyses were made if possible. The primary analysis population was a full analysis set, which included all study subjects. No imputation procedures were applied on missing data. The ethical approval was obtained from the ethics committee of the Hospital District of Southwest Finland and the study was reported to the National Supervisory Authority for Welfare and Health. The study was monitored regularly. The study was conducted according to the clinical investigation plan, ISO 14155 and Good Clinical Practice.

4.2.2 Preparation of samples (III-V)

A combination of 1 g bioactive glass and clodronate in the form of dinatrium salt (200 mg (**III**, **V**); 100 mg, 200 mg and 300 mg (**IV**)) was used as dry powder and with 900 µl (**III**- **V**) or 1350 µl (**IV**) of 0.9% saline added. Bioactive glass needs a liquid medium to start the bioactive process. 0.9% saline alone is a weak inducer

of this process and apatite formation. Therefore, in order to better study the properties of the combination, and minimize the effect of added medium, the liquid medium chosen was 0.9% saline. Additionally, the normal clinical practice, while using BAG as a bone filler, is to moisturize it with saline before use (BonAlive 2012). For some analyses these wetted samples were dried just before measurement at room temperature on top of a filter paper until no visual sign of moisture was seen on the paper (approximately 10 minutes).

Clodronate (0.1255 g, 5.76×10^{-4} mol) and calcium hydroxide (0.0439 g, 5.93×10^{-4} mol; equivalent mole amounts) were mixed with a small quantity of distilled water (~5 mL) and heated for 2 min. The resulting thick white paste was left to cool overnight. The calcium salt of clodronate was filtered off using a sintered glass funnel, washed with distilled water and acetone and left to air dry with a 93% yield obtained (0.1027 g, 5.35×10^{-4} mol) (III).

4.2.3 Particle size measurement and pH (III, IV)

The particle size distribution (PSD) of the granules was determined using a 3D-surface image analysis method (Flashsizer FS3D, Intelligent Pharmaceuticals Ltd, Helsinki, Finland). Approximately 3 mm³ of both clodronate powder and bioactive granules were analyzed by FS3D. This technique is described in details by Soppela et al. (2011). pH measurements (Fieldlab pH, L7137A, Schott Instruments, Germany) of the wetted samples (with 900 µl of 0.9% saline) were preformed over time up to day 25. pH was recorded at the following time points: every half hour for the first two hours, every second hour for next six hours, every 24th hour until day 4, and then once each on days 7, 8, 10, 15, 25. The measurement time points were based on earlier reported results for bioactive glasses (Hench and Andersson, 1999).

4.2.4 Scanning electron microscopy (III, IV)

Scanning electron micrographs (SEM) were used to visualize the changes in morphology between the samples. SEM is a type of electron microscope, which uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimen and produces images of a sample based on these signals. Samples were attached to a double-sided carbon tape and coated with 20 nm platinum using an Agar sputter coater B7304 (Agar Scientific Ltd., Stansted, UK). Electron micrographs were taken by scanning electron microscopy (DSM962, Zeiss, Oberkochen, Germany and FEI Quanta 250 FEG, Holland).

4.2.5 Differential scanning calorimetry (III, IV)

Differential scanning calorimetry (DSC) is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a

sample is measured as a function of temperature. It gives information about physical transformation such as phase transitions and physical changes, such as glass transitions. DSC measurements were performed on a Mettler DSC analyser (model 821e, Mettler Toledo Ag, Schwerzenbach, Switzerland) using STAR software (STAR 5.1, Sun Soft Inc., Mountain View, CA, USA). The temperature axis of the equipment was calibrated with zinc and indium. The runs were performed under nitrogen gas flow (50 mL/min) in aluminium pans, and the weights of the samples were 5-7 mg. The heating rate was 10 °C/min over the temperature range 15 to 450°C.

4.2.6 X-ray powder diffractometry (III, IV)

X-ray powder diffraction (XRPD) use X-ray for structural characterization of materials. It gives information *e.g.*, about phase identity of materials, degree of crystallinity, primary determinant of polymorphism and phase composition of mixtures. The X-ray diffraction patterns of the dry and wetted samples were measured using an XRPD 2theta diffractometer (Brukeraxs D8, Germany). The XRPD experiments were performed in symmetrical reflection mode with CuK α radiation (1.54 Å) using Göbel Mirror bent gradient multilayer optics. The scattered intensities were measured with a scintillation counter. The angular range was from 5 to 40° (2 θ) (III) and 20 to 40° (2 θ) (IV) with steps of 0.05° (III) and 0.04° (IV) and the measuring time was 6 s/step (III) and 1 s/step (IV). For the different BAG fraction comparison the measurements were done repeated with five times (IV). Bruker Analytical X-ray systems, Diffrac plus EVA structure database was used to help in identification. All of the samples were measured at room temperature.

4.2.7 Fourier transform Raman spectroscopy (III)

Raman spectra were taken using the Bruker Equinox 55 interferometer with the FRA 106/S Raman accessory. Fourier transform Raman spectroscopy is a spectroscopic technique used to observe vibrational, rotational, and other low-frequency modes in a system, and to get information *e.g.*, the chemical bonds and symmetry of molecules. The Raman scattering was generated using 1064 nm Nd:YAG laser and scattered light was detected using a D418-T liquid nitrogen cooled Ge-detector. Measurements were taken at 200 mW power, with 128 scans per spectrum produced with 2 cm⁻¹ resolution and a large spot size of ~1 mm diameter to avoid sub sampling. Each sample was compacted into two divets with three measurements taken from each divet. The OPUS 5.0 software (Bruker Optik) was used for data collection and OPUS 6.5 (Bruker Optik) was used for spectral manipulations. Clodronate sample with saline solution was measured at 300 mW with a ~300 μ m spot size in a liquid cell; all other parameters were as described above.

4.2.8 Fourier transform infrared spectroscopy (III, IV)

Fourier transform infrared (FTIR) spectroscopy was used to characterize the composition of the samples. It obtains an infrared spectrum of absorption and transmission scattering of a sample. Attenuated total reflectance infrared (ATR-IR) spectroscopy was used to analyze dry and wetted samples. The samples were measured using a Bruker Alpha-P ATR-IR (Bruker Optics Inc., MA, USA) with 48 scans per spectrum at 2 cm^{-1} resolution with each sample measured three times. For better contact against the ATR crystal, samples with large grains (contain BAG) were crushed slightly using a mortar and pestle. Spectra were collected and analyzed (averaged and peak picking) using Bruker OPUS 6.5 software (Bruker Optics Inc. MA, USA).

4.2.9 Focused-ion beam (FIB) and energy dispersive X-ray spectroscopic (EDS) mapping (IV)

The reaction interface of the BAG and clodronate combination was studied with a FEI Quanta 3D 200i DualBeam FIB-SEM (Oregon, USA). FIB system use a finely focused beam of ions to generate a variety of signals at the surface of solid specimen and produces images of a sample based on these signals. A 10 nm Au/Pd conductive coating was prepared with Cressington sputter coater (UK). Standard FIB-SEM lift-out procedure was then used to extract a 2 micron thick specimen cross-section foil from the sample surface. The thin section sample was attached on its other side on a piece of single crystal silicon in order to improve electrical contact for energy-dispersive X-ray spectrometry (EDS) mapping. An energy-dispersive (EDS) detector was used to separate the characteristic X-rays of different elements into an energy spectrum Low-vacuum EDS mapping in 0.3 mbar chamber pressure was used for studying the outer surface and glass exposed through exfoliation of a section of the dried surface. Both cross-section high vacuum and top-down low vacuum EDS analysis were done with a 10 kV electron probe, which is a compromise between spatial resolution and efficient X-ray generation from calcium. EDS data were collected with a Xmax 50 mm² SSD X-ray spectrometer in the FIB-SEM instrument. Data analysis was done with an Oxford Instruments Inca 350 software (UK).

5 Results and discussion

5.1 The effect of oral adjuvant clodronate on bone mineral density (I) and incidence of bone metastases (II)

The results indicate that patients treated for primary operable breast cancer have evidence of bone loss, as estimated by DEXA (dual energy X-ray absorptiometry) measurements of bone mineral density. In premenopausal patients clodronate significantly reduced the loss of bone mineral density at one year, although this effect was not seen at two years (Table 4). This result confirms the similar findings from another smaller study as well as a study conducted with risedronate (Delmas et al. 1997, Saarto et al. 1997). In contrast, postmenopausal patients on placebo had relatively little bone loss. This is probably because most of these patients received the adjuvant tamoxifen, which has been shown to reduce the bone loss in postmenopausal women (Love et al. 1992). In postmenopausal women on clodronate, there was a statistically significant increase in spinal BMD at one and two years.

Table 4. *Change in spinal BMD (%) in premenopausal and postmenopausal breast cancer patients after one and two years receiving either clodronate or placebo.*

	Clodronate	Placebo	p-value
Premenopausal			
1 y	-1.57	-4.04	0.003
2 y	-3.99	-3.94	0.9
Postmenopausal			
1 y	1.63	-0.37	0.003
2 y	2.00	0.09	0.04

Even after dividing the data into subgroups based on study sites, menopausal status or adjuvant tamoxifen treatment, the treatment effect remained in favour of clodronate. In particular, the bone loss was significant after one year in premenopausal and perimenopausal women who received placebo treatment.

Premenopausal breast cancer women have a higher risk of bone loss because their primary treatment for breast cancer is chemotherapy, which induces bone loss (Bruning et al. 1990). Additionally, most of the premenopausal women received tamoxifen, which acts like an antiestrogen and thus causes bone loss (Powles et al. 1996). The finding that clodronate could statistically significantly reduce the bone loss in premenopausal women during first year, but the effect was not seen any more at second year is understandable, because primary breast cancer treatment which indirectly causes bone loss was given mostly during the first year only.

Patients with radiologically confirmed bone metastases totaled 143 during the studied period. Out of these, 63 were in the clodronate group and 80 in the placebo controlled group. This finding was not statistically significant. During the study medication period (first two years of the trial), there was a significant reduction in the occurrence of bone metastases for patients on clodronate compared to placebo (12 vs 28). This result was not seen for the total follow-up period (Fig. 8). The finding is in line with results reported by Diel et al. (1998, Diel 2001) in patients with primary breast cancer with detected micrometastases in the bone marrow.

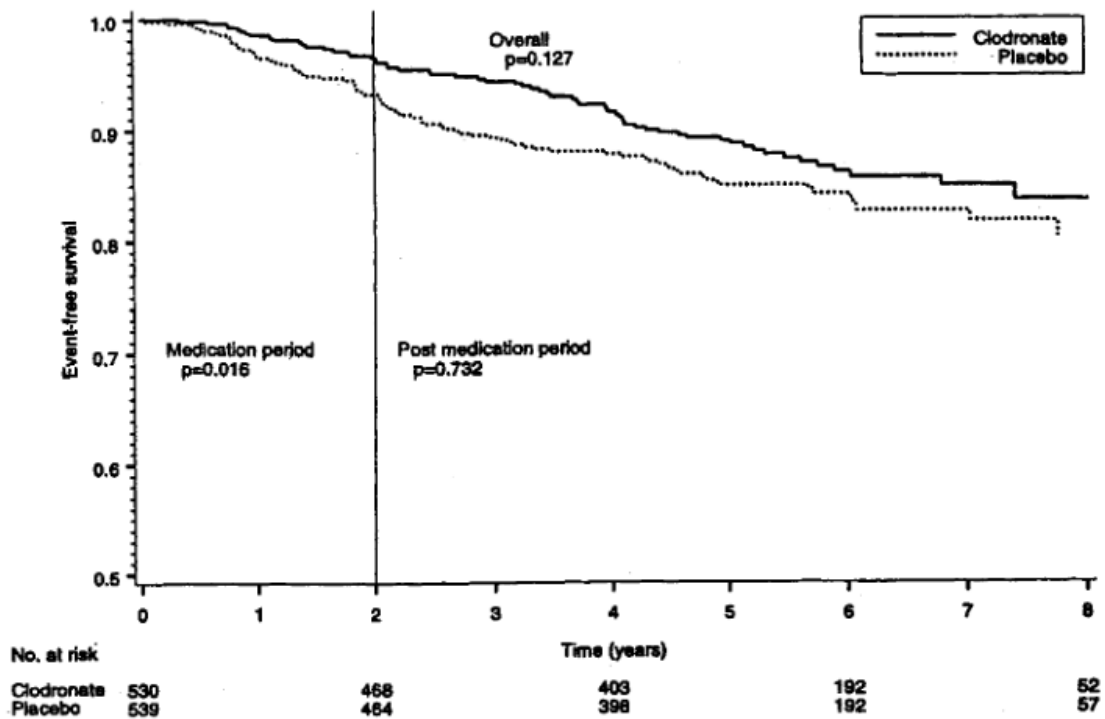


Figure 8. Kaplan-Meier curve for bone metastase- free survival during medication and post medication period in primary breast cancer patients receiving either oral clodronate or placebo. Number of patients included to the analysis shown at the lower part of the figure.

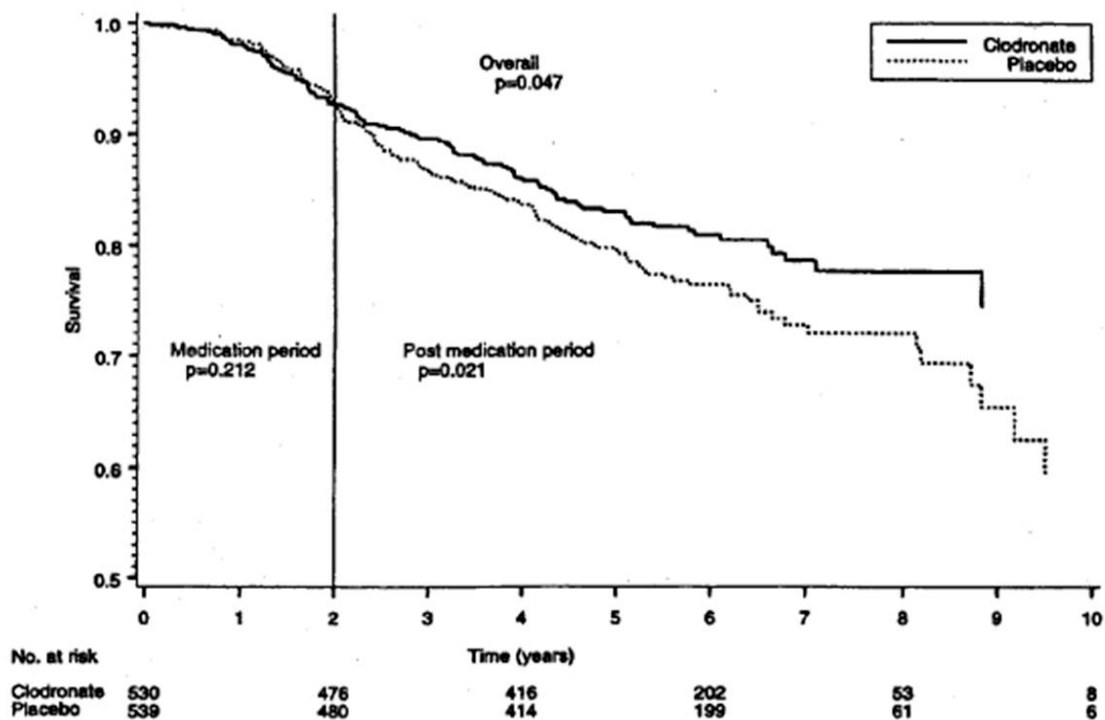


Figure 9. Kaplan Meier curve for deaths occurring during medication and post medication period in primary breast cancer patients receiving either oral clodronate or placebo. Number of patients included to the analysis shown at the lower part of the figure.

There was a significant reduction in mortality for patients receiving clodronate (Fig. 9). During the whole study period there were 98 deaths reported in the clodronate group whereas there were 129 in the placebo group ($p=0.047$). The effect for mortality was seen more clearly during the follow-up period ($p=0.021$).

As bone metastases are one of the main factors for poor prognosis in cancer, it follows that, if the bone can be “saved” from bone metastases the survival is better. In this study this hypothesis was proven. This study was the first one to report the effect of adjuvant clodronate therapy for survival. Subsequently, there have been several other studies confirming the result, for example a study by Diel et al. (2008). Potential confounding factors such as type of tumor, method of surgery, radiation and systemic cancer treatment were evenly distributed between the study groups and did not affect the results of our study.

In the study by Diel et al. (1998) the decreased occurrence of other than bone metastases was reported. In our study this was not observed. There was no difference in occurrence of other than bone metastases either during the study medication period or follow-up period between the groups. There were 38 other metastases reported in the clodronate group during the study medication period and 74 at follow-up (total 112). The corresponding numbers for the placebo group were 39 and 89, respectively (total 128).

Treatments in both the clodronate and placebo groups were well tolerated. The significant side effects for patients on clodronate were an increased occurrence of diarrhea and a decreased occurrence of skin rash. The occurrence of diarrhea is similar to that reported in several other studies (Hannuniemi et al. 1991, Mian et al. 1991, Diel et al. 2007). It is due to the very high oral doses needed to achieve the desired blood level of clodronate because of poor bioavailability. In the subgroup of the BMD study there were 27 patients in the clodronate and 8 patients in the placebo group reporting diarrhea and in the total study population the numbers were 88 vs 40 respectively ($p < 0.001$). In the clodronate treated patients, there was a significantly lower incidence of rash ($p < 0.01$), which is a typical adverse effect for cancer treatments. This could be due to the anti-inflammatory effect of clodronate mediated through the effect on macrophage function (Makkonen et al. 1999). Otherwise, clodronate was well tolerated and even among prematurely terminated patients there was no difference in reported adverse events between the study groups.

There are several cost-effectiveness studies done including clodronate as long term therapy in malignancy. Even there are differences in these studies e.g., treatment time, cancer type and treatment, possible metastases associated and even the most studies have not collected quality of life information, it is evident that clodronate is cost-effective adjuvant cancer treatment. Especially this is shown in mean costs of skeletal complications, which reduced by 50% in four years of treatment in myeloma patients (McCloskey et al. 2005).

5.2 Development of delivery system for local administration of clodronate (III, IV)

5.2.1 Characterization of the interaction between bioactive glass and clodronate (III, IV)

The combination of bioactive glass and clodronate was characterized using pH and particle size measurement, scanning electron microscopy, differential scanning calorimetry, X-ray powder diffractometry, Fourier transform Raman and infrared spectroscopy.

Depending on the method used for particle size measurement the BAG samples having the bigger particle size fraction had different outcomes. Based on the measurements using 3D-surface image analysis, particles were up to 1320 μm in a

single dimension and more than 50% of the particles in the sample had a dimension larger than 750 μm . The manufacturer of the bioactive glass claims that the particle size of the sample is between 500-800 μm . The difference in data is likely to be due to the manufacturer using sieve analysis for the particle size distribution measurement. In sieve analysis particles are selected based on two dimensions allowing the particle to go through the hole in the sieve. However, the third dimension of the particle can exceed the indicated size of the sieve. However, it is important to understand that particle size dimension can be bigger than that stated by the manufacturer. The total surface area of the BAG granules is the area in which the bioactivity, i.e. exchange of ions, is occurring. Thus, particle size is important. The same kind of difference related to the analytical method was not observed in the samples containing the smaller particles.

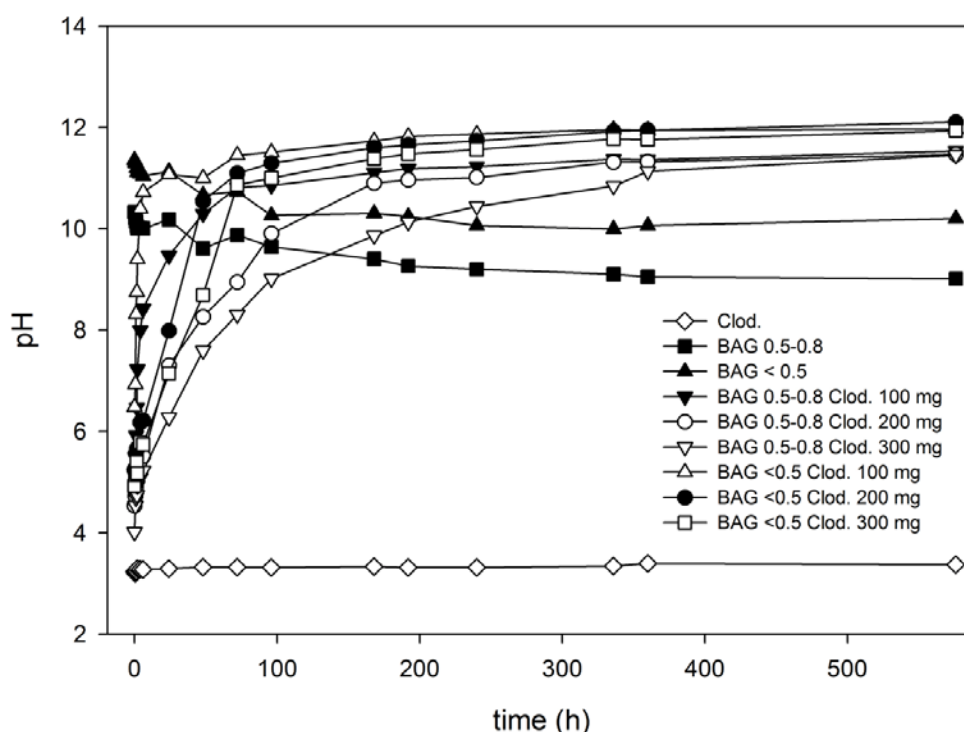


Figure 10. Changes in pH over time (h) for clodronate (Clod.), bioactive glass (BAG) 0.5-0.8 mm or <0.5 mm and combination product (BAG (0.5-0.8 mm or <0.5 mm) + Clod. (100 mg, 200mg or 300mg)) with saline 1350 μl .

The increase in pH was higher for the combination of BAG and clodronate than BAG alone, which indicates that the activity of the BAG is stronger in the combination product. Additionally, the elevated pH was sustained for longer time in the combination product than BAG alone. The apatite formation on the top of

the bioactive glass is related to the ion exchange that causes the pH increase (Hench and Andersson 1999). The pH change seen in both BAG products (particle size <0.5 mm and 0.5-0.8 mm) is similar to that reported by Hench and Andersson as well as Kokubo (Hench and Andersson 1999, Kokubo 1999), *i.e.*, rapid ion change resulting in an increase in pH which gradually becomes steady. This phenomenon can be seen in Figure 10. The greater the degree of ion exchange, which is reflected as an increase in pH, the greater the inferred bioactivity. As seen in Figure 10, as expected, the activity is greater in the BAG with a smaller particle size both in pure bioactive glass and in the combination formulation. However, the differences in the combination products are not that prominent. The difference in pH with as a function of clodronate amount is not clear either. Surprisingly, it seems that a greater amount of clodronate leads to lower activity (*e.g.* less ion exchange) when comparing the different BAG particle size groups and the amount of clodronate. As seen in Figure 10, the combination product with 300 mg clodronate for both BAG fraction groups has the lowest pH (least active) the whole way through the experiment. This phenomenon can be explained with results involving clodronate calcium precipitation formation (III). As more clodronate calcium precipitation is formed (the more clodronate), bioactivity appears to decrease.

Raman spectra (Fig. 11) together with FTIR spectra provide clear evidence that there is a strong interaction between the bioactive glass and clodronate resulting in an enhanced ion exchange. The interaction seems to be that of an extended ion exchange between the BAG and clodronate, resulting in a layer of calcium clodronate forming on the surface of the bioactive glass (Fig. 11). This kind of interaction can be explained. It is known that in an acidic environment glass granules release Ca^{2+} ions, which bind with OH^- groups (Yli-Urpo et al. 2004). As clodronate solution decreases the pH around the combination mixture, the release of Ca^{2+} ions is promoted. Moreover, the affinity of clodronate to Ca^{2+} is well known (Fleisch et al. 1970). Thus, the layer on the top of the bioactive glass could indeed be solid calcium clodronate. However, the possibility that the formed layer includes CaP, as described by Oliveira et al. (2010) cannot be totally excluded. The spectrum of the bioactive glass combined with clodronate and saline showed a strong crystalline peak at 1014 cm^{-1} which does not correspond to the ν_1 phosphate symmetric stretching mode from apatite ($952\text{-}985\text{ cm}^{-1}$, Sauer et al. 1994, Penel et al. 1998) and its Raman shift is too low to be a mineral carbonate ($1053\text{-}1099\text{ cm}^{-1}$ (Herman et al. 1987, Thomas et al. 2007)). When comparing the Raman spectra of BAG, clodronate and saline with mono, di and tri-calcium phosphate, the sharp peak most closely resembles that of dicalcium phosphate, suggesting that one possible configuration is -PO_3^{2-} binding to Ca^{2+} (Fig. 12a) and another possibility is two HPO_3^- groups binding to Ca^{2+} (Fig. 12b).

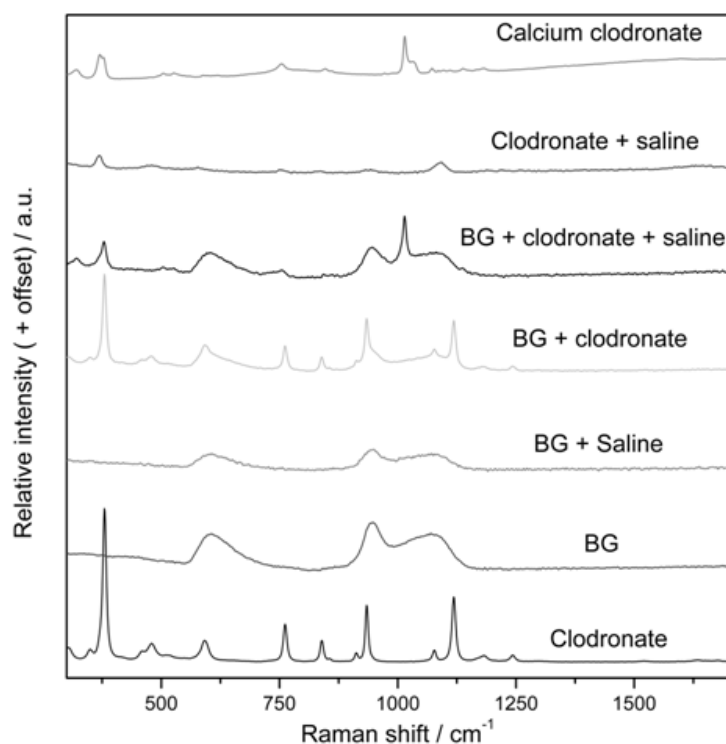


Figure 11. Averaged Raman spectra of the samples.

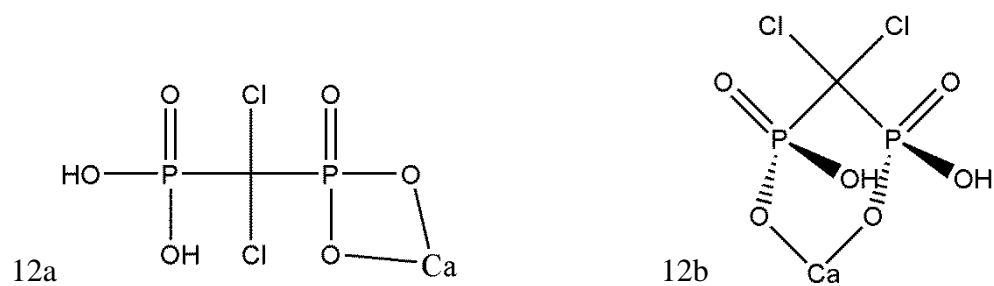


Figure 12. Possible complexes formed when bioactive glass and clodronate are wetted with saline solution.

With SEM micrographs the changes in morphology can be detected whereas DSC thermograms give information about possible changes in crystal form (lattice). Both SEM and DSC results confirmed the results obtained with pH measurement and Fourier transform spectroscopy (Raman and infrared), which

suggest that the combination product has greater bioactivity. The SEM micrograph suggests that there is more layer formation on the top of the bioactive glass with the combination product than BAG alone. However, DSC shows that the perturbation caused by the presence of clodronate is not a simple addition of calcium clodronate, but a more complex interaction (Fig. 13). One explanation could be that as described by Hench and Andersson (1999) in stage 4 (reaction of bioactive glass) there is migration of Ca^{2+} needed. Knowing that clodronate promotes this (as there is great affinity of clodronate to calcium), this would facilitate formation of the $\text{CaO-P}_2\text{O}_5$ - rich layer. But if there is too much clodronate available, this enhancement of Ca^{2+} migration would lead to calcium clodronate precipitation. In fact, the SEM images indicate that the interpretation using pH to suggest greater bioactivity with less clodronate seems to also fit the BAG granule size. As revealed by DSC, the combination results cannot be explained purely with calcium clodronate precipitation. Thus the layer formation on the top of the BAG is not purely calcium clodronate, but could also be a combination of calcium clodronate and apatite formation. One could assume, the smaller the particle size the greater the reaction surface and thus the higher the bioactivity. Conversely, we found that the layer formation may be more extensive in the combination product with the larger granule size. This would mean that, as there is a larger reaction surface on which to form calcium clodronate, similar to the amount of clodronate present; the bioactivity of the BAG would be reduced.

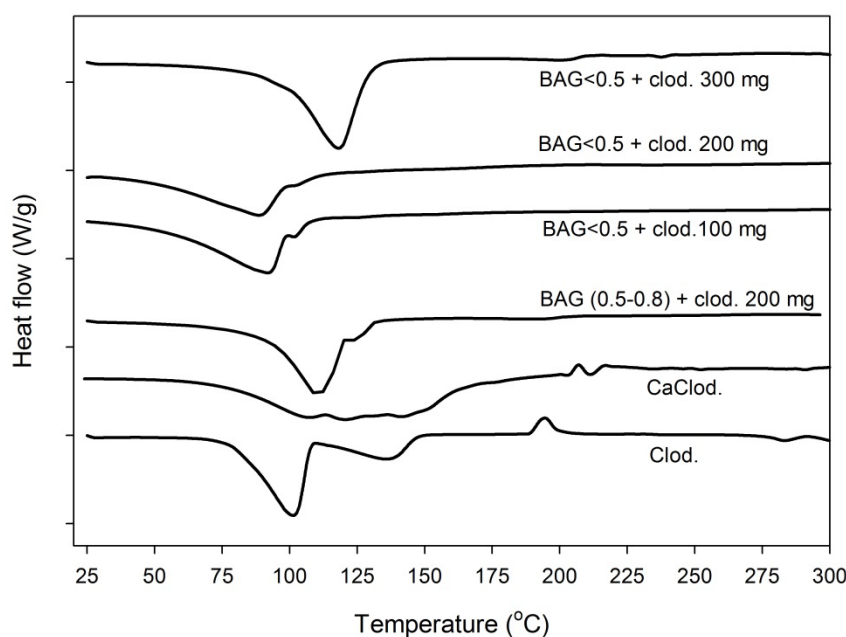


Figure 13. DSC thermograms of clodronate (Clod.), calcium clodronate (CaClod.) and combination products of bioactive glass (BAG) with two particle size fractions (<0.5 mm and 0.5-0.8 mm) and different amounts of clodronate (Clod.) interacted with saline for 72 hours.

The interaction of bioactive glass and clodronate was also characterized with XRPD. The diffraction pattern from the combination of BAG and clodronate sample does not match that of clodronate, however many of the diffraction peaks (such as $(2\theta) = 24^\circ\text{--}26^\circ$, $28^\circ\text{--}30^\circ$ and $32^\circ\text{--}34^\circ$) are similar to the signal from calcium clodronate. The XRPD of combination product also shows features that may be attributed to apatite formation (Chen et al. 2010, Oliveira et al. 2010, Ravarian et al. 2010, Mneimne et al. 2011). Additionally, Ca_2SiO_4 (around $(2\theta) = 33.9^\circ$) was clearly seen indicating the activity of the bioactive glass. In stage 4 of the activity of the bioactive glass (indicating apatite formation), it is typical that Ca^{2+} and PO_4^{3-} groups migrate to the surface through the SiO_2 -rich layer, $(2\theta) = 32^\circ\text{--}33^\circ$ (Hench and Andersson 1999, Oliveira et al. 2010). It seems also that some calcium silicate chloride is formed, as evidenced by peaks at $(2\theta) = 28.9^\circ$, 35.4° and 30.3° . While comparing the two different BAG fractions (particle size <0.5 mm and $0.5\text{--}0.8$ mm) in the combination product with 200 mg clodronate, it is apparent that BAG with the smaller particle size did not differ much in bioactivity from the BAG with the larger particle size, as would have been expected (Fig. 14).

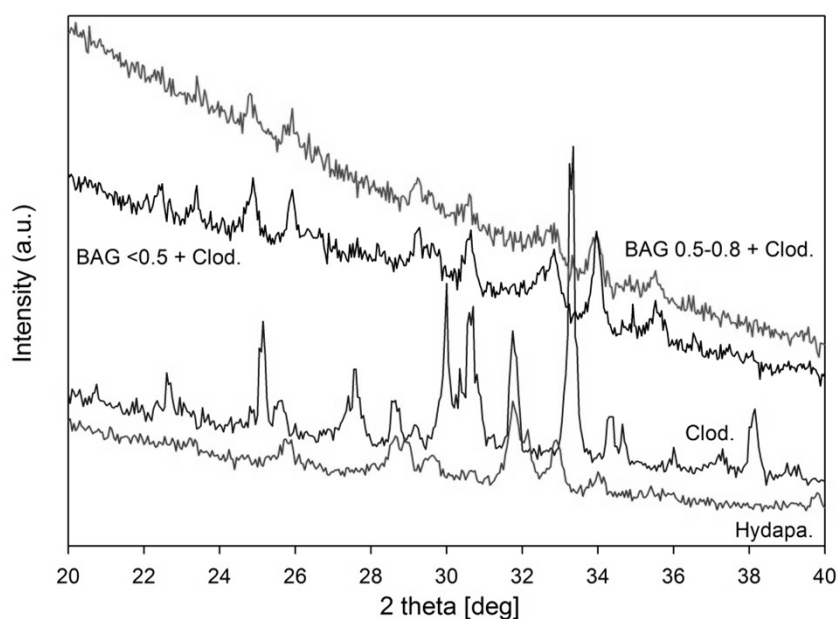


Figure 14. XRPD diffractogram of pure clodronate (Clod.), hydroxyapatite (Hydapa.) and combination product (BAG+Clod. (200mg clodronate)) with two different BAG fractions (BAG <0.5 mm and BAG $0.5 - 0.8$ mm) interacted with saline for 48 hours.

Based on all the results (with pH measurement, SEM, DSC, XRPD, Raman, FTIR and FIB and EDX spectroscopic mapping), it is apparent that there is a strong interaction between bioactive glass and clodronate resulting in an

enhanced ion exchange. The interaction seems to be such that an extended ion exchange between the BAG and clodronate results in a layer of calcium clodronate forming on the surface of the bioactive glass. However, the possibility that the formed layer includes CaP as described by Oliveira et al. (2010) cannot be excluded. This theory is supported by pH, X-ray diffractometry and SEM results. Most importantly, the understanding is that clodronate enhances the bioactivity of bioactive glass and both calcium clodronate precipitation and apatite are formed. The amount of apatite formation and level of bioactivity of BAG depends on the amount of clodronate and particle size of BAG. The greater the amount of clodronate present, the greater the formation of calcium clodronate— but this happens at the expense of apatite formation. Equally, the more there is surface available to react, the greater the calcium clodronate precipitation and the less the apatite formation. Finally, it can be argued that some of the earlier results presented by Oliveira et al. (2010) could have been different, if those had been compared to the calcium clodronate as well, but obviously there was also apatite formation included.

5.2.2 Evaluating optimal combination of clodronate and bioactive glass for dental application (IV)

As one of the aims of this study was to select the best combination of clodronate and BAG for local application in the treatment of periodontitis, the results of the characterization studies were evaluated. Even if the increase in pH was greater in the BAG with the smaller particle size both in pure BAG and combination product, it could be noted that the bioactivity was not necessary better. In our opinion, both elevated ion exchange and apatite formation ability are considered as desired bioactivity. As shown in SEM micrographs and DSC thermograms and confirmed with XRPD diffractogram FTIR and FIB-EDS, it is important to choose the correct ratio of clodronate and BAG to enhance the activity of the BAG, but at the same time not to have too much calcium clodronate precipitation, which might lower the apatite formation. According to the same principles, the BAG fraction with the smaller particle size (<0.5 mm) and thus more reactive surface facilitates calcium clodronate formation more than apatite. To maximize the bioactivity of BAG enhanced by clodronate, there should be a limited amount of calcium clodronate precipitation, which allows apatite formation. At the same time, the pH in the mouth should be kept as near as possible to the normal value of pH 7. With high pH values the gingiva gets irritated. Therefore, for dental application the most suitable BAG and clodronate combination product of those studied would be the one with lowest pH with the best ability to form apatite. The correct amount of clodronate would be that which enhances the bioactivity of the BAG, best apatite formation ability and amount of clodronate enough to enhance the bioactivity of the BAG and allow apatite formation.

When considering the optimal amount of clodronate, it was concluded that 300 mg was too much as too much calcium clodronate precipitation occurred. The

properties in smaller amounts of clodronate (100 mg and 200 mg) did not differ much with respect *e.g.*, to amount of calcium clodronate formation. Therefore, since the maximum enhancement of bioactivity of BAG was one of the targets, the 200 mg dose was chosen. The BAG fraction with the smaller particle size reacted too strongly, which could be seen for example as too high pH values. Therefore the fraction of 0.5-0.8 mm was chosen. Based on these considerations, the combination product of 200 mg clodronate and BAG with particle size 0.5-0.8 mm was chosen as the most promising formulation for dental application to treat periodontitis. The selection was made based on pH results and observed solid state properties of the combination.

5.3 Novel bioactive glass – clodronate combination as an adjunctive agent at periodontal maintenance phase (V)

The selected combination product was compared to the bioactive glass alone in the treatment of the periodontitis maintenance phase in a pilot study with ten patients (2 female and 8 male). Their mean age was 66.5 years and their mean BMI was 26 (mildly overweight). Out of 10 subjects, seven participated in all scheduled visits and eight completed the follow-up.

The PPD values of the treated teeth reduced significantly during the study for both the BAG and combination product, while no differences in VPI or BOP values were observed. Gingival recession (the apical movement of the gingival tissue boundary) increased significantly for both treatment options from baseline to follow-up visit 2. The PAL decreased significantly in the combination product treated teeth at both follow-up visits as compared to the baseline, whereas with the BAG treated teeth a significant result was only seen at the last follow-up visit.

For three subjects, the teeth to be treated with BAG or combination product differed slightly at baseline, as the reported value of pain after cold water and air blow. At follow-up visit 2, all subjects estimated the situation with combination product treated teeth to be better than with the BAG treated teeth. At every visit from the second visit onwards, patients were asked to evaluate their situation compared to that before treatment. The following scale was used: excellent (1), good (2), satisfactory (3), poor (4). The mean evaluation was slightly better for the combination product than BAG at each visit. However, the median value was the same for both treatment options (Table 5). During both follow-up visits, the evaluation for combination product treated teeth varied from excellent to good and for BAG treated teeth from excellent to satisfactory (first follow-up) or even poor (second follow-up).

Table 5. Mean and median score for study subject's (n=number of subjects) evaluation for the situation compared to that at visit before (excellent (1), good (2), satisfactory (3), poor (4)) in teeth treated with either combination product of bioactive glass and clodronate (BAG+Clod.) or bioactive glass (BAG) alone.

Visit	BAG+Clod. (mean, median [min-max])		BAG (mean, median [min-max])	
Treatment visit 2 (n=10)	2.40	2 [2-4]	2.50	2 [2-4]
Treatment visit 3 (n=9)	2.11	2 [2-3]	2.33	2 [2-4]
Treatment visit 4 (n=8)	2.00	2 [1-3]	2.25	2 [1-4]
Follow-up visit 1 (n=8)	1.88	2 [1-2]	2.00	2 [1-3]
Follow-up visit 2 (n=8)	1.88	2 [1-2]	2.13	2 [1-4]
Total (n=8)	2.05	2 [1-4]	2.24	2 [1-4]

Starting from the second treatment visit, the subjects were asked to report their overall satisfaction (better, unchanged, or worse) since the previous visit. There were no differences between the treatment options for overall satisfaction until the last follow-up visit. The subjects reported the situation to be slightly better in teeth treated with the combination product (median: BAG+clodronate “better” vs. BAG “unchanged”).

During the study, samples indicating bone metabolism were collected for biomarker analysis. There was a significant change ($p<0.05$) in the amount of OPG in BAG treated teeth at both follow-up visits when compared to the baseline value (Fig. 15), while in teeth treated with combination product there was a significant reduction in the amount of OPG only between last follow-up and the baseline. A significant difference was observed between the treatment options at first follow-up visit when teeth treated with BAG had lower OPG levels than those treated with the combination product. Osteocalcin values remained at low levels throughout the study for both treatment options.

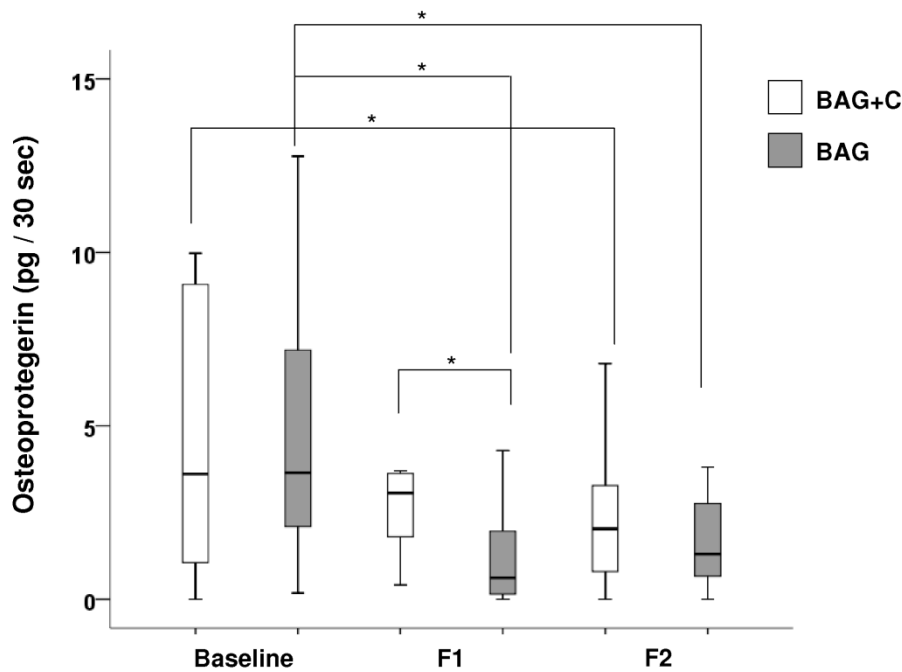


Figure. 15 Total amount of osteoprotegerin in collected gingival crevicular fluid (GCF) (pg / 30 sec) from the test (bioactive glass and clodronate, BAG+C) and control (bioactive glass, BAG) site at baseline, first follow-up visit (F1) and second follow-up visit (F2). The box blots present the median (horizontal line), quartiles (boxes) and non-outlier values (whiskers). * $p < 0.05$

There was only one adverse event recorded during the study. One subject had a mucosal lesion at the gingival area of a combination product treated tooth after the first treatment period and this led to premature termination of the study. The situation was recovered within a month. Otherwise both treatment options were well tolerated and there were no safety issues reported. The single adverse event could have several causes, especially since it did not appear right after the treatment. However, the possibility of study treatment causing this one event cannot be ruled out.

The limitations of our study include the restricted number of participants, rather few applications of test and control agents, and a relatively short follow-up period. Compared to earlier investigations done with BAG, for example, Biosilicate® (fully crystallized bioactive glass) in the treatment of hypersensitive teeth (Tirapelli et al. 2011), the Biosilicate® treatment was given twice daily for 30 days, e.g., daily and more often than in our study. Therefore, it was surprising that with the limited amount of treatments administered in the current study, such

good results were observed. For instance, the reduction of pain scores was similar to the above mentioned study (Tirapelli et al. 2011) and the reduction in pain lasted after the treatment completion to the follow-up visits.

Based on previously published data, it was obvious that BAG alone is beneficial also in the given indication. Several study groups have published the advantageous effects of bioactive glass in clinical signs of periodontal disease, such as decreased probing depth and increased clinical attachment levels with an appropriate control arm (Ong 1998, Pandit et al. 2010, Yadav et al. 2011). A recently conducted meta-analysis supports this information (Sohrabi et al. 2012). Therefore, there was no need for a control group without any treatment, and the teeth treated with bioactive glass alone served as a suitable control group for the combination BAG and clodronate product treatment. The pathogens from the biofilm and the content of saliva are known to have an important role in periodontitis and subject-to-subject variation. In this study, subjects acted as their own comparator, thus reducing this confounding factor.

Two previous studies involving the combination of bisphosphonate and bioactive glass as a local treatment have been mentioned in the Introduction (Välimäki et al. 2006, Srisubut et al. 2007). These studies differ from the current study, as in both studies BP and bioactive glass were administered simultaneously without making a combination product. Additionally, the administration route was different; the BP was given subcutaneously or the mixture was used as a filling material. In both studies the results concluded beneficial effects for bone. In the current literature, there are several animal studies on the potential of using bisphosphonates for the management of periodontitis or preventing root resorption when moving teeth by orthodontic means. In an experimental study on periodontitis in rats, Mitsuta et al. (2002) suggested that the local administration of clodronate may be effective in preventing osteoclastic activity leading to bone resorption, which is the main characteristic of periodontitis. Another bisphosphonate, alendronate, was examined in a beagle dog model for its capability of inhibiting alveolar bone loss (Reddy et al. 1995). In the alendronate group, a significant increase in bone mass was observed compared to placebo groups. In a rat study by Yaffe et al. (2003), where local delivery of alendronate was used together with tetracycline, a combined effect was demonstrated in the reduction of alveolar bone loss. Furthermore, as a potent blocker of bone resorption, risedronate has been examined in the context of orthodontic (*i.e.* dental displacement) tooth movements in rats (Adachi et al. 1994, Igarashi et al. 1996); it was suggested that the local administration of risedronate has favorable effects by preventing root resorption.

The results of this study, reported in this thesis, suggest that local administration of clodronate may be effective in preventing osteoclastic bone resorption in periodontitis. Similar to above listed studies we could see some indication that, based on biomarker data obtained, the amount of alveolar bone loss was less in the teeth treated with the combination product (BAG+clodronate) than those treated with BAG alone. However, due to the short time period of

investigation as well as the limited number of subjects and treated teeth, the result remains only indicative.

Additionally, the selected biomarkers might not be the ones reflecting inhibition of osteoclastic bone resorption. Osteoprotegerin is known to inhibit the differentiation activity of osteoclasts by blocking receptor activator of nuclear factor-kappa B (RANK) ligand (RANKL) (Kirkwood et al. 2007). It is thus a good biomarker for bone resorption and bone loss. Bisphosphonates cause osteoblasts to release osteoprotegerin (Viereck et al. 2002). Clodronate is known to inhibit osteoclast activity through apoptosis (Frith et al. 2001) and, further, COX-2 dependent prostaglandin E2 production and RANKL expression in periodontal ligament cells (Liu et al. 2006). Since the release of OPG was examined from sites with nitrogen containing bisphosphonates in these published studies, the possible effect for decreased bone degradation may not be seen so clearly in the investigation using OPG as a biomarker. Moreover, even if it is known that the bioactive glass release ionic products and enhance the expression of osteogenic markers like osteocalcin (Varanasi et al. 2011, Tousi et al. 2013), it was not possible to demonstrate that in this study. Another consideration is that the amount of clodronate may not have been enough to cause a pharmacological effect. When administered locally, the amount of clodronate is some 12 % of that given systemically (1600 mg daily). Therefore, the effect of clodronate in the amount used in the combination product of this study is merely based on physicochemical properties and reactions enhancing the activity of bioactive glass, than other mechanisms. Additionally, as the amount used to enhance bioactivity is small the cost of the agent is not limiting for its use for this kind of application.

6. Conclusions

This study confirms that use of oral clodronate is safe and effective in preventing bone loss in patients who have been treated for primary operable breast cancer. The cost benefits of widespread use of clodronate (or other bisphosphonate) as an intervention for patients receiving systemic treatment for primary breast cancer has been discussed and at the moment the guidelines recommend adjuvant bisphosphonate therapy (Winter and Coleman 2013). Moreover, for bone loss the best advantage from clodronate was achieved in pre- and perimenopausal women with breast cancer. In postmenopausal women the reduction in bone loss was not so pronounced, which can be attributed to tamoxifen treatment. Unlike in premenopausal women, tamoxifen is known to increase bone density in postmenopausal women (Powles et al. 1996). Therefore, it can be concluded that breast cancer patients benefitting the most from clodronate treatment would be pre- and perimenopausal women with breast cancer that has advanced beyond stage I.

Bioactivity expressed as ion exchange of the BAG and combination products have been clearly shown with using pH measurement, SEM micrographs, XRPD, Raman and FTIR spectroscopy as well as FIB and EDX spectroscopic mapping. Calcium clodronate formation in the combination product was demonstrated using SEM and XRPD, but was most clear with Raman and FTIR. Furthermore, based on pH data the ion exchange is more extensive in the combination product than BAG alone. There are also signs of apatite formation, as shown with typical peaks of apatite in X-ray powder diffractograms and IR spectra. Thus, it can be concluded that clodronate enhances the ion exchange of the BAG. On the other hand, based on *e.g.* pH data, the ion exchange occurs for a longer period in the combination product than BAG alone. This suggests that the combination of clodronate and BAG creates a more favorable environment than BAG alone for ion exchange and thus bioactivity, and clodronate has a remarkable ability to enhance the activity of BAG. This could also be beneficial in clinical applications. An important consideration is whether this kind of combination in clinical use should be applied in such a way that ion exchange has already started outside the human body, *i.e.*, first introduced in saline, rather than directly in the physiological environment. As explained earlier, the ion exchange generally leads to an increase in solution pH, which can be substantial for finely grained powders having high surface to volume ratios (Hench and Wilson 1999, Felipe et al. 2009). The creation of a very alkaline environment can cause cell and neuron damage and therefore, ion exchange prior to application should help to minimize the possible harmful effects.

In order to study the local treatment of periodontitis in a clinical setting, the best combination product was defined. When one of the definitions for bioactivity is apatite formation, it could be concluded, that too much increased ability to

form calcium clodronate precipitation has negative influence and should be minimized. Calcium clodronate levels that were considered too high were believed to have occurred with the highest amount of clodronate, *i.e.*, 300 mg, and BAG with the smallest particle size (and largest surface area for reaction). The combination product of 200 mg clodronate and BAG with particle size 0.5-0.8 mm was selected as the most promising formulation for dental application to treat periodontitis. Additionally, it can be concluded that the effect of clodronate and BAG in the combination product is not restricted to the chemical properties alone; the outcome was also influenced by physical properties that allows multiple opportunities to vary composition and physical structure enhance the therapeutic action.

Based on the results of the pilot clinical study the combination product of clodronate and BAG seems to be at least as good as BAG alone in the treatment of periodontitis in the maintenance phase. The results did not differ between the treatments as evaluated in general clinical signs such as VPI or BOP. Individually evaluating the other variables also failed to reveal a substantial difference between the treatments. However, when all variables were considered (*e.g.* PPD, OPG, overall satisfaction, pain after cold water and air blow as well as subjective evaluation) together, the combination product had slightly better results. Therefore, it seems that even if the traditional clinical signs or biomarkers of bone did not show any clear difference between the treatments, a more holistic analysis suggests that adding clodronate to the BAG will enhance the already known favorable effects of BAG alone.

Overall, it can be concluded that in the primary breast cancer patient population the positive effect of clodronate on bone was evident and in local administration for periodontitis treatment in combination with BAG the results on bone effects indicated positive treatment effects. The bioactivity of BAG was enhanced by clodronate and this could be verified to some extent also in the clinical setting. In conclusion, based on these results, it is safe to continue the development of the combination product of clodronate and BAG. The combination product needs to be further refined in order to obtain a final clinically useful preparation with an applicator in the treatment of periodontitis, and possibly other conditions such as hyper-sensitive teeth and bone defects.

References

- Adachi H, Igarashi K, Mitani H, Shinoda H. Effects of topical administration of a bisphosphonate (risedronate) on orthodontic tooth movements in rats. *J Dent Res* 1994;73:1478-1486.
- American Cancer Society. *Cancer Facts & Figures 2013*. Atlanta: American Cancer Society; 2013.
- American Dental Association (ADA) Council on Scientific Affairs. Dental management of patients receiving oral bisphosphonate therapy: expert panel recommendations. *J Am Dent Assoc* 2006;137:1144-1150.
- Andersson ÖH, Kangasniemi I. Calcium phosphate formation at the surface of bioactive glass in vitro. *J Biomed Mater Res* 1991;25:1019-1030.
- Andersson ÖH, Vähätalo K, Yli-Urpo A, Happonen R-P, Karlsson K. Short-term reaction kinetics of bioactive glass in simulated body fluid and subcutaneous tissue. In Andersson ÖH, Happonen R-P, Yli-Urpo A. (Eds.), *Biocheramics*. Cambridge, Butterworth-Heinemann 1994;7:67-72.
- Banerjee A, Hajatdoost-Sani M, Farrell S, Thompson I. A clinical evaluation and comparison of bioactive glass and sodium bicarbonate air-polishing powders. *J Dent* 2010 38:475–479.
- Berenson JR, Rosen L, Vescio R, HS, Woo M, Sioufi A, Kowalski MO, Knight RD, Seaman JJ. Pharmacokinetics of pamidronate disodium in patients with cancer with normal or impaired renal function. *J Clin Pharmacol* 1997;37:285-290.
- Bergkvist L, Tabár L, Lau Adami HO, Persson I, Bergström R. Mammographic parenchymal patterns in women receiving noncontraceptive estrogen treatment. *Am J Epidemiol* 1989;130:503-510.
- Bianco AR, Del Mastro L, Gallo C, Perrone F, Matano E, Pagliarulo C, De Placido S. Prognostic role of amenorrhea induced adjuvant chemotherapy in premenopausal patients with early breast cancer. *Br J Cancer* 1991;63:799-803.
- Binderman I, Adut M, Yaffe A. Effectiveness of Local Delivery of Alendronate in Reducing Alveolar Bone Loss Following Periodontal Surgery in Rats. *J Periodontol* 2000;71:1236-1240.
- Blomqvist C. Malignant hypercalcaemia, a hospital survey. *Acta Med Scand* 1986;220:455-463.
- BonAlive® granules, CE 0344. BonAlive Biomaterials Ltd. Instructions for use. 2012.
- Boskey AL. Mineralization of bones and teeth. *Elements* 2007;3:387-393.
- Bruning PF, Pit MJ, de Jong-Bakker M, van den Ende A, Hart A, van Enk A. Bone mineral density after adjuvant chemotherapy for premenopausal breast cancer. *Br J Cancer* 1990;61:308-310.
- Brunsvold MA, Chaves ES, Kornman KS, Aufdemorte TB, Wood R. Effects of a bisphosphonate on experimental periodontitis in monkeys. *J Periodontol* 1992;63:825-830.
- Buencamino MC, Palomo L, Thacker HL. How menopause affects oral health, and what we can do about it. *Cleve Clin J Med* 2009;76:467-475.
- Castren-Kortekangas P, Löyttyniemi E, Liukko-Sipi S, Juhakoski A, Smal J, Laitinen K. Pooling of clodronate urinary excretion data: a new

- pharmacokinetic method to study drugs with highly variable gastrointestinal absorption. *J Bone Miner Res* 1997;12:66-71.
- Carey JJ, Palomo L. Bisphosphonates and osteonecrosis of the jaw: innocent association or significant risk? *Cleve Clin J Med* 2008;75:871-879.
- Chavassieux PM, Arlot ME, Reda C, Wei L, Yates AJ, Meunier PJ. Histomorphometric assessment of the long-term effects of alendronate on bone quality and remodeling in patients with osteoporosis. *J Clin Invest* 1997;100:1475-1480.
- Chen C-C, Wang W-C, Ding S-J. In vitro physicochemical properties of a biomimetic gelatin/chitosan oligosaccharide/calcium silicate cement. *J Biomed Mater Res Part B: Appl Biomater* 2010;95B:456-465.
- Clarke B. Normal bone anatomy and physiology. *Clin J Am Soc Nephrol* 2008;3:131-139.
- Conrad KA, Lee SM. Clodronate kinetics and dynamics. *Clin Pharmacol Ther* 1981;30:114-120.
- Delmas PD, Balena R, Confravreux E, Hardouin C, Hardy P, Bremond A. Bisphosphonate risedronate prevents bone loss in women with artificial menopause due to chemotherapy of breast cancer: a double-blind, placebo-controlled study. *J Clin Oncol* 1997;15:955-962.
- Diel IJ. Bisphosphonates in the prevention of bone metastases: Current evidence. *Semin Oncol* 2001;4:75-80.
- Diel IJ, Solomayer EF, Costa SD, Gollan C, Goerner R, Wallwiener D, Kaufmann M, Bastert G. Reduction in new metastases in breast cancer with operable primary breast cancer. *N Engl J Med* 1998;339:357-363.
- Diel IJ, Bergner R, Grötz KA. Adverse effects of bisphosphonates: current issues. *J Support Oncol* 2007;5:475-82.
- Diel IJ, Jaschke A, Solomayer EF, Gollan C, Bastert G, Sohn C, Schuetz F. Adjuvant oral clodronate improves the overall survival of primary breast cancer patients with micrometastases to the bone marrow: a long-term follow-up *Ann Oncol* 2008;19:2007-2201.
- Dodson TB, Raje NS, Caruso PA, Rosenberg AE. Case records of the Massachusetts General Hospital. Case 9-2008. A 65-year-old woman with a nonhealing ulcer of the jaw. *N Engl J Med* 2008;358:1283-1291.
- Elonen E, Wiklund T. Chemotherapy. In: Joensuu H, Roberts PJ, Teppo L. (Eds.), *Cancer disease*. Duodecim, Helsinki, 1999 pp. 135-164.
- Elte JWF, Bijvoet OLM, Cleton FJ, van Oosterom AT, Sleeboom HP. Osteolytic bone metastases in breast carcinoma pathogenesis, morbidity and bisphosphonate treatment. *Eur J Cancer Clin Oncol* 1986;22:439-500.
- El-Shinnawi UM, El-Tantawy SI. The effect of alendronate sodium on alveolar bone loss in periodontitis (clinical trial). *J Int Acad Periodontol* 2003;5:5-10.
- Ezra A, Golomb G. Administration routes and delivery systems of bisphosphonates for the treatment of bone resorption. *Adv Drug Deliv Rev* 2000;42:175-195.
- Faulkner KG, Cummings SR, Black D. Simple measurement of femoral geometry predicts hip fracture; the study of osteoporotic fractures. *J Bone Miner Res* 1993;8:1211-1217.
- Felipe ME, Andrade PF, Novaes AB Jr., Grisi MFM, Souza SLS, Taba M Jr., Palioto DB. Potential of Bioactive Glass Particles of Different Size Ranges to Affect Bone Formation in Interproximal Periodontal Defects in Dogs. *J Periodontol* 2009;80:808-815.

- Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JWW, Comber H, Forman D, Bray F. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012. *Eur J Cancer* 2013;49:1374–1403.
- Filho OP, LaTorre GP, Hench LL. Effect of crystallization on apatite layer formation of bioactive glass S45P5. *J Biomed Mater Res* 1996;30:509-514.
- Filipponi P, Pedetti M, Fedeli L, Cini L, Palumbo R, Boldrini S, Massoni C, Cristallini S. Cyclical clodronate is effective in preventing postmenopausal bone loss: a comparative study with transcutaneous hormone replacement therapy. *J Bone Miner Res* 1995;10:697-703.
- Fleisch H. Bisphosphonates: Pharmacology and use in the treatment of tumour-induced hypercalcaemic and metastatic bone disease. *Drugs* 1991;42:919-944.
- Fleisch H. Bisphosphonates in bone disease: From the laboratory to the patient. Parthenon, Athens, London, 1997 pp. 11-30, 57-62, 115-137.
- Fleisch HA, Russell RGG, Bisaz S, Muhlbauer RC, Williams DA. The inhibitory effect of phosphonates on the formation of calcium phosphate crystals in vitro and on aortic and kidney calcification in vivo. *Eur. J. Clin. Invest* 1970;1:12–18.
- Fonong T, Burton DJ, Pietrzyk DJ. Determination of formation constants of calcium complexes of difluoromethylenediphosphonic acid and related diphosphonates. *Anal Chem* 1983 55:1089-1094.
- Francis MD. The inhibition of calcium hydroxyapatite crystal growth by polyphosphonates and polyphosphates. *Calcif. Tissue Res* 1969;3:151–162.
- Francis MD, Russell RG, Fleisch H. Diphosphonates inhibit formation of calcium phosphate crystals in vitro and pathological calcification in vivo. *Science* 1969;165:1262–1264.
- Frith JC, Mönkkönen J, Auriola S, Mönkkönen H, Rogers MJ. The molecular mechanism of action of the antiresorptive and anti-inflammatory drug clodronate: evidence for the formation in vivo of a metabolite that inhibits bone resorption and causes osteoclast and macrophage apoptosis. *Arthritis Rheum* 2001;44: 2201-2210.
- Fromigie O, Kheddoumi N, Body JJ. Bisphosphonates antagonize bone growth factors effects on human breast cancer cells survival. *Br J Cancer* 2003;89:178–184.
- Garnero P, Grimaux M, Seguin P, Delmas PD. Characterisation of immunoreactive forms of human osteocalcin generated in vivo and in vitro. *J Bone Miner Res* 1994;9:255-264.
- Giannini S, D'Angelo A, Malvasi L, Castrignano R, Pati T, Tronca R, Liberto L, Nobile M, Crepaldi. G Effects of one-year cyclical treatment with clodronate on postmenopausal bone loss. *Bone* 1993;14:137-141.
- Gillam DG, Tang JY, Mordan NJ, Newman HN. The effects of a novel Bioglass® dentifrice on dentine sensitivity: a scanning electron microscopy investigation. *J Oral Rehabil* 2002;29:305-313.
- Giuliani N, Pedrazzoni M, Passeri G, Girasole G. Bisphosphonates inhibit IL-6 production by human osteoblast-like cells. *Scand J Rheumatol* 1998;27:38-41.
- Green JR. Antitumor effects of bisphosphonates. *Cancer* 2003;97:840–847.
- Hannuniemi R, Laurén L, Puolijoki H. Clodronate: an effective agent for the treatment of increased bone resorption. *Drugs of Today* 1991;27:375-390.
- Hansen NM, Felix R, Bisaz S, Fleisch H. Aggregation of hydroxyapatite crystals. *Biochim. Biophys. Acta* 1976;451:549–559.
- Hayakawa S, Tsuru K, Ohtsuki C, Osaka A. Mechanism of apatite formation on a sodium silicate glass in a simulated body fluid. *J Am Ceram Soc* 1999;82:2155-2160.

- Healey JH. Metastatic cancer to the bone. In: De Vita VT, Hellman S, Rosenberg SA (Eds.), *Cancer, Principles & practice of oncology*. Lippincott-Raven publishers, Philadelphia, 1997 pp. 2570-2586.
- Hench LL, Andersson Ö. Bioactive glasses. In: Hench LL, Wilson J. (Eds.), *Advanced Series in Ceramics vol1. - An introduction to bioceramics*. World Scientific Publishing Co.Pte.Ltd., Singapore, 1999 pp. 41-62.
- Hench LL, Paschall HA. Histochemical responses at a biomaterials interface. *J Biomed Mater Res Symp* 1974;5:49-64.
- Hench LL, Wilson J. Surface-active biomaterials. *Science* 1984;226:630-636.
- Hench LL, Wilson J. Introduction. In: Hench LL, Wilson J. (Eds.), *Advanced Series in Ceramics vol1. - An introduction to bioceramics*. World Scientific Publishing Co.Pte.Ltd., Singapore, 1999 pp. 1-24.
- Herman RG, Bogdan CE, Sommer AJ, Simpson DR. Discrimination among Carbonate Minerals by Raman-Spectroscopy Using the Laser Microprobe. *Appl Spectrosc* 1987;41:437-440.
- Hughes DE, MacDonald BR, Russell RG, Gowen M. Inhibition of osteoclast-like cell formation by bisphosphonates in long-term cultures of human bone marrow. *J Clin Invest* 1989;83:1930-1935.
- Hughes DE, Wright KR, Uy HL, Sasaki A, Yoneda T, Roodman GD, Mundy GR, Boyce BF. Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo. *J Bone Miner Res* 1995;10:1478-1487.
- Igarashi K, Adachi H, Mitani H, Shinoda H. Inhibitory effect of the topical administration of a bisphosphonate (risedronate) on root resorption incident to orthodontic tooth movement in rats. *J Dent Res* 1996;75:1644-1649.
- Isidor F, Karring T, Attström R. Reproducibility of pocket depth and attachment level measurements when using a flexible splint. *J Clin Periodontol* 1984;11:662-668. a
- Isidor F, Karring T, Attström R. The effect of root planning as compared to that of surgical treatment. *J Clin Periodontol* 1984;11:669-681. b
- Jeffcoat MK, Cizza G, Shih WJ, Genco R, Lombardi A. Efficacy of bisphosphonates for the control of alveolar bone loss in periodontitis. *J Int Acad Periodontol* 2007;9:70-76.
- Kanis JA. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: Synopsis of a WHO report. *Osteoporosis Int* 1994;4:368-381.
- Kanis JA, Melton LJ III, Christiansen C, Johnston CC, Khaltsev N. Perspective: the diagnosis of osteoporosis. *J Bone Miner Res* 1994;9:1137-1141.
- Kanis JA, Powles T, Paterson AH, McCloskey EV, Ashley S. Clodronate decreases the frequency of skeletal metastases in women with breast cancer. *Bone* 1996;19:663-667.
- Kataja V, Johansson R. Basics for hormonal therapy. In: Joensuu H, Roberts PJ, Teppo L. (Eds.), *Cancer disease*. Duodecim, Helsinki, 1999 pp. 165-177.
- Kellokumpu-Lehtinen P. Biological treatment. In: Joensuu H, Roberts PJ, Teppo L. (Eds.), *Cancer disease*. Duodecim, Helsinki, 1999 pp. 178-190.
- Kirkwood KL, Cirelli JA, Rogers JE, Giannobile WV. Novel host response therapeutic approaches to treat periodontal diseases. *Periodontol* 2000 2007;43:294-315.
- Kokubo T. A/W glass –ceramic: processing and properties. In: Hench LL, Wilson J. (Eds.), *Advanced Series in Ceramics vol1. - An introduction to bioceramics*. World Scientific Publishing Co.Pte.Ltd., Singapore, 1999 pp. 75-88.

- Kokubo T, Takadama H. How useful is SBF in predicting in vivo bone activity? *Biomaterials* 2006;27:2907-2915.
- Korhonen LK, Väänänen HK. Bone inductive molecules and enhancement of repair and regeneration. In Hall BK. (Eds.), *Bone*. CRP Press, Boca Raton, 1992 pp. 209-231.
- Kormano M. Bone, joints and muscles. In: Strandertskjöld-Nordenstam C-G, Kormano M, Laasonen EM, Soimakallio S, Suramo I. (Eds.), *Clinical Radiology*. Duodecim, Helsinki, 1998 pp. 123-224.
- Kouri M, Valavaara R. Effect of radiation to normal tissue and tolerance dose. In: Lahti T, Holsti LR. (Eds.), *Clinical radiation biology*. Duodecim, Helsinki, 1997 pp. 143-174.
- Krejci CB, Bissada NF. Periodontitis--the risks for its development. *Gen Dent* 2000;48:430-436.
- Kröger H, Huopio J, Honkanen R, Tuppurainen M, Puntila E, Alhava E, Saarikoski S. Prediction of fracture risk using axial bone mineral density in a premenopausal population: a prospective study. *J Bone Miner Res* 1995;10:302-306.
- Kröger H, Kotaniemi A, Vainio P, Alhava E. Bone densitometry of the spine and femur in children by dual-energy X-ray absorptiometry. *Bone Miner* 1992;17:75-85.
- Kuo TC, Lee BS, Kang SH, Lin FH, Lin CP. Cytotoxicity of DP-bioglass paste used for treatment of dentin hypersensitivity. *J Endod* 2007;33:451-454.
- Laitinen K, Patronen A, Harju P, Löyttyniemi E, Pylkkänen L, Kleimola T, Perttunen K. Timing of food intake has a marked effect on the bioavailability of clodronate. *Bone* 2000;27:293-296.
- Liberman UA, Weiss SR, Bröll J, Minne HW, Quan H, Bell NH, Rodriguez-Portales J, Downs RW Jr, Dequeker J, Favus M. Effect of oral alendronate on bone mineral density and the incidence of fractures in postmenopausal osteoporosis. The Alendronate Phase III Osteoporosis Treatment Study Group. *N Engl J Med* 1995;333:1437-1443.
- Lindfors NC, Heikkilä JT, Koski I, Mattila K, Aho AJ. Bioactive glass and autogenous bone as bone graft substitutes in benign bone tumors. *J Biomed Mater Res B Appl Biomater* 2009;90:131-136.
- Lindfors NC, Hyvönen P, Nyyssönen M, Kirjavainen M, Kankare J, Gullichsen E, Salo J. Bioactive glass S53P4 as bone graft substitute in treatment of osteomyelitis. *Bone* 2010;47:212-218.
- Liu L, Igarashi K, Kanzaki H, Chiba M, Shinoda H, Mitani H. Clodronate inhibits PGE₂ production in compressed periodontal ligament cells. *J Dent Res* 2006;85:757-760.
- Lockyer MWG, Holland D, Dupree R. NMR investigation of the structure of some bioactive and related glasses. *J Non-Cryst Solid* 1995;188:207-219.
- Love RR, Mazzees RB, Barden HS, Epstein S, Newcomb PA, Jordan VC, Carbone PP, DeMets DL. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *N Engl J Med* 1992;326:852-856.
- Makkonen N, Salminen A, Rogers MJ, Frith JC, Urtti A, Azhayeva E, Mönkkönen J. Contrasting effects of alendronate and clodronate on RAW 264 macrophages: the role of a bisphosphonate metabolite. *Eur J Pharm Sci* 1999;8:109-118.
- Marshall D, Johnell O, Wedel H. Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. *Br Med J* 1996;312:1254-1259.

- McCloskey EV, Guest JF, Ashford RU, Kanis JA. Cost implications of bisphosphonates, in: Jasmin C, Capanna R, Coleman RE, Coia LR, Saillant G. (Eds.), *Textbook of Bone Metastases*. John Wiley & Sons Ltd. West Sussex, England, 2005 pp. 415-426.
- Meyer JL, Nancollas GH. The influence of multidentate organic phosphonates on the crystal growth of hydroxyapatite. *Calcif Tissue Res* 1973;13:295–303.
- Mian M, Beghé F, Caprio A, Aloj R, Bertelli A. Tolerability and safety of clodronate therapy in bone diseases. *Int J Clin Pharmacol Res* 1991;11:107-114.
- Mitsuta T, Horiuchi H, Shinoda H. Effects of topical administration of clodronate on alveolar bone resorption in rats with experimental periodontitis. *J Periodontol* 2002;73:479-486.
- Mneimne M, Hill RG, Bushby AJ, Brauer DS. High phosphate content significantly increases apatite formation of fluoride-containing bioactive glasses. *Acta Biomater* 2011;7:1827-1834.
- Montazeri AH, Erskine JG, McQuaker IG. Oral sodium clodronate induced osteonecrosis of the jaw in a patient with myeloma. *Eur J Haematol* 2007;79:69-71.
- Mundy GR. Pathophysiology of cancer-associated hypercalcemia. *Semin Oncol* 1990;17:10-15.
- National Institute of Health and Welfare. Dental health of adults in Finland. Health 2000 survey. 2004.
- Ong MM, Eber RM, Korsnes MI, MacNeil RL, Glickman GN, Shyr Y, Wang HL. Evaluation of a bioactive glass alloplast in treating periodontal intrabony defects. *J Periodontol* 1998;69:1346-1354.
- Oliveira AL, Pedro AJ, Saiz Arroyo C, Mano JF, Rodriguez G, San Roman J, Reis RL Biomimetic Ca-P coatings incorporating bisphosphonates produced on starch-based degradable biomaterials. *J Biomed Mater Res Appl Biomater* 2010; 92B, 55-67.
- Padmanabhan N, Wang DY, Moore JW, Rubens RD. Ovarian function and adjuvant chemotherapy for early breast cancer. *Eur J Cancer Clin Oncol* 1987;23:745-748.
- Pajus I, Lestang P, Lioté F, Dryll A. Erythroderma after clodronate treatment. *BMJ* 1993;307:484.
- Palomo L, Bissada N, Liu J. Periodontal assessment of postmenopausal women receiving risedronate. *Menopause* 2005;12:658-660.
- Palomo L, Liu J, Bissada NF. Skeletal bone diseases impact the periodontium: a review of bisphosphonate therapy. *Expert Opin Pharmacother* 2007;8:309-315.
- Pandit N, Gupta R, Gupta S. A comparative evaluation of biphasic calcium phosphate material and bioglass in the treatment of periodontal osseous defects: a clinical and radiological study. *J Contemp Dent Pract* 2010;11:25-32.
- Parfitt AM. Bone remodelling: relationship to the amount and structure of bone, and the pathogenesis and prevention of fractures. In: Riggs BL, Melton III LJ. (Eds.), *Osteoporosis: etiology, diagnosis, and management*. Raven Press, New York, 1988 pp. 45-93.
- Paterson AH, Powles TJ, Kanis JA, McCloskey E, Hanson J, Ashley S. Double-blind controlled trial of oral clodronate in patients with bone metastases from breast cancer. *J Clin Oncol* 1993;11:59-65.
- Peltola M, Aitasalo K, Suonpää J, Varpula M, Yli-Urpo A. Bioactive glass S53P4 in frontal sinus obliteration: a long-term clinical experience. *Head Neck* 2006 28: 834–841.

- Penel G, Leroy G, Rey C, Bres E. MicroRaman spectral study of the PO₄ and CO₃ vibrational modes in synthetic and biological apatites. *Calcif Tissue Int* 1998;63:475-481.
- Pentikäinen PJ, Elomaa I, Nurmi A-K, Kärkkäinen S. Pharmacokinetics of clodronate in patients with metastatic breast cancer *Int J Clin Pharmacol Ther Toxicol* 1989;27:222-228.
- Plosker GL, Goa KL. Clodronate: A review of its pharmacological properties and therapeutic efficacy in resorptive bone disease. *Drugs* 1994 47:945-982.
- Pouilles J, Tremollieres F, Bonneau M, Ribot C. Influence of early age at menopause on vertebral bone mass. *J Bone Miner Res* 1994;9:311-315.
- Powles TJ, Hickish T, Kanis JA, Tidy A, Ashley S. Effect of tamoxifen on bone mineral density measured by dual-energy X-ray absorptiometry in healthy premenopausal and postmenopausal women. *J Clin Oncol* 1996;14:78-84.
- Ravarian R, Moztaaradeh F, Solati Hashjin M, Rabiee SM, Khoshakhlagh P, Tahriri M. Synthesis, characterization and bioactivity investigation of bioglass/hydroxyapatite composite. *Ceramics Int* 2010;36: 291-297.
- Reddy MS, Weatherford TW 3rd, Smith CA, West BD, Jeffcoat MK, Jacks TM. Alendronate treatment of naturally-occurring periodontitis in beagle dogs. *J Periodontol* 1995;66:211-217.
- Reinhardt RA, Payne JB, Maze CA, Patil KD, Gallagher SJ, Mattson JS. Influence of estrogen and osteopenia/osteoporosis on clinical periodontitis in postmenopausal women. *J Periodontol*. 1999;70:823-828.
- Richards MA, O'Reilly SM, Howell A, George WD, Fentiman IS, Chaudary MA, Growther D, Rubens RD. Adjuvant cyclophosphamide, methotrexate, and fluorouracil in patients with axillary node-positive breast cancer: An update of the Guy's/Manchester trial. *J Clin Oncol* 1990;8:2032-2039.
- Risteli L, Risteli J. Biochemical markers of bone metabolism. *Ann Med* 1993;25:385-393.
- Ritch PS. Treatment of cancer-related hypercalcemia. *Semin Oncol* 1990;17:26-33.
- Robey PG. Biochemistry of bone. In: Riggs BL, Melton III LJ. (Eds.), *Osteoporosis*. Lippincott-Raven, Philadelphia, 1995 pp. 41-83.
- Rocha ML, Malacara JM, Sánchez-Marin FJ, Vazquez de la Torre CJ, Fajardo ME. Effect of alendronate on periodontal disease in postmenopausal women: a randomized placebo-controlled trial. *J Periodontol* 2004;75:1579-1585.
- Rogers MJ, Gordon S, Benford HL, Coxon FP, Luckman SP, Monkkonen J, Frith JC. Cellular and molecular mechanisms of action of bisphosphonates. *Cancer* 2000;88:2961-2978.
- Rolla G, Bucca C, Brussino L. Bisphosphonate-induced bronchoconstriction in aspirin-sensitive asthma. *Lancet*. 1994;343:426-427.
- Ruggiero SL, Mehrotra B, Rosenberg TJ, Engroff SL. Osteonecrosis of the jaws associated with the use of bisphosphonates: a review of 63 cases. *J Oral Maxillofac Surg* 2004;62:527-534.
- Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B; Task Force on Bisphosphonate-Related Osteonecrosis of the Jaws, American Association of Oral and Maxillofacial Surgeons. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaw - 2009 update. *Aust Endod J* 2009;35:119-130.
- Saarto T, Blomqvist C, Ehnholm C, Taskinen MR, Elomaa I. Effects of chemotherapy induced castration on serum lipids and apoproteins in premenopausal women with node-positive breast cancer. *J Clin Endocrinol Metab* 1996;81:4453-4457.

- Saarto T, Blomqvist C, Välimäki M, Mäkelä P, Sarna S, Elomaa I. Chemical castration induced by adjuvant cyclophosphamide, methotrexate, and fluorouracil chemotherapy causes rapid bone loss that is reduced by clodronate: a randomized study in premenopausal breast cancer patients. *J Clin Oncol* 1997;15:1341-1347.
- Saha H, Castrén-Kortekangas P, Ojanen S, Juhakoski A, Tuominen J, Tokola O, Pasternack A. Pharmacokinetics of clodronate in renal failure. *J Bone Miner Res* 1994;9:1953-1958.
- Sahni M, Guenther HL, Fleisch H, Collin P, Martin TJ. Bisphosphonates act on rat resorption through the mediation of osteoblasts. *J. Clin. Invest* 1993;91:2004–2011.
- Santini D, Vespasiani Gentilucci U, Vincenzi B, Picardi A, Vasaturo F, La Cesa A, Onori N, Scarpa S, Tonini G. The antineoplastic role of bisphosphonates: from basic research to clinical evidence. *Ann Oncol* 2003;14:1468–1476.
- Sato M, Grasser W. Effects of bisphosphonates on isolated rat osteoclasts as examined by reflected light microscopy. *J Bone Miner Res* 1990;5:31-40.
- Sauer GR, Zunic WB, Durig JR, Wuthier RE. Fourier-Transform Raman-Spectroscopy of Synthetic and Biological Calcium Phosphates. *Calcif Tissue Int* 1994;54:414-420.
- Shoji K, Horiuchi H, Shinoda H. Inhibitory effects of a bisphosphonate (risedronate) on experimental periodontitis in rats. *J Periodontal Res* 1995;3:277-284.
- Sohrabi K, Saraiya V, Laage TA, Harris M, Blieden M, Karimbux N. An evaluation of bioactive glass in the treatment of periodontal defects: a meta-analysis of randomized controlled clinical trials. *J Periodontol* 2012;83:453-64.
- Soppela I, Airaksinen S, Hatara J, Räikkönen H, Antikainen O, Yliruusi J, Sandler N. Rapid particle size measurement using 3D surface imaging. *AAPS Pharm Sci Tech* 2011;12, 476-484.
- Srisubut S, Teerakapong A, Vattraphodes T, Taweekaisupapong S. Effect of local delivery of alendronate on bone formation in bioactive glass grafting in rats. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2007;104,11-16.
- Stewart AF, Horst R, Deftos LJ, Cadman EC, Lang R, Broadus AE. Biochemical evaluation of patients with cancer-associated hypercalcemia: evidence for humoral and nonhumoral groups. *N Engl J Med* 1980;303:1377-1383.
- Stoor P, Söderling E, Grénman R. Bioactive Glass S53P4 in Repair of Septal Perforations and Its Interactions with the Respiratory Infection-Associated Microorganisms *Haemophilus influenzae* and *Streptococcus pneumoniae* *J Biomed Mater Res (Appl Biomater)* 2001;58:113–120.
- Stoor P, Pulkkinen J, Grénman R. Bioactive glass S53P4 in filling of cavities in the mastoid cell area in surgery for chronic otitis media. *Ann Otol Rhinol Laryngol* 2010;119:377-382.
- Tezal M, Wactawski-Wende J, Grossi SG, Ho AW, Dunford R, Genco RJ. The relationship between bone mineral density and periodontitis in postmenopausal women. *J Periodontol* 2000;71:1492-1498.
- Thomas DB, Fordyce RE, Frew RD, Gordon KC. A rapid, non-destructive method of detecting diagenetic alteration in fossil bone using Raman spectroscopy. *J Raman Spectrosc* 2007;38:1533-1537.
- Tirapelli C, Panzeri H, Lara EH, Soares RG, Peitl O, Zanotto ED. The effect of a novel crystallised bioactive glass-ceramic powder on dentine hypersensitivity: a long-term clinical study. *J Oral Rehabil J Oral Rehabil* 2011;38:253-262.

- Tuominen U, Salonen J, Happonen R-P, Yli-Urpo A. Effect of bioactive glass granules and polytetrafluoroethylene membrane on repair of cortical bone defect. *J Mater Sci: Mater in Med* 1995;6:639-641.
- Tousi NS, Velten MF, Bishop TJ, Leong KK, Barkhordar NS, Marshall GW, Loomer PM, Aswath PB, Varanasi VG. Combinatorial effect of Si^{4+} , Ca^{2+} , and Mg^{2+} released from bioactive glasses on osteoblast osteocalcin expression and biomineralization. *Mater Sci Eng C Mater Biol Appl* 2013;33:2757-2765.
- Turunen T, Peltola J, Makkonen T, Helenius H, Yli-Urpo A. Bioactive glass granules and polytetrafluoroethylene membrane in the repair of bone defects adjacent to titanium and bioactive glass implants. *J Mater Sci Mater Med* 1998;9:403-407.
- Urwin GH, Yates AJ, Gray RE, Hamdy NA, McCloskey EV, Preston FE, Greaves M, Neil FE, Kanis JA. Treatment of the hypercalcemia of malignancy with intravenous clodronate. *Bone* 1987;8:43-51.
- Van Gelder JM, Breuer U, Ornoy A, Schlossman A, Patlas N, Golomb G. Anticalcification and antiresorption effects of bisacylphosphonates. *Bone* 1995;16:511-520.
- Varanasi VG, Owyong JB, Saiz E, Marshall SJ, Marshall GW, Loomer PM. The ionic products of bioactive glass particle dissolution enhance periodontal ligament fibroblast osteocalcin expression and enhance early mineralized tissue development. *J Biomed Mater Res Part A* 2011;98A:177-184.
- Viereck V, Emons G, Lauck V, Frosch KH, Blaschke S, Gründker C, Hofbauer LC. Bisphosphonates pamidronate and zoledronic acid stimulate osteoprotegerin production by primary human osteoblasts. *Biochem Biophys. Res Commun* 2002;291:680-686.
- Villikka K, Perttunen K, Rosnell J, Ikävalko H, Vaho H, Pylkkänen L. The absolute bioavailability of clodronate from two different oral doses. *Bone* 2002;31:418-421.
- von Wowern N, Klausen B, Kollerup G. Osteoporosis: a risk factor in periodontal disease. *J Periodontol* 1994;65:1134-1138.
- Välimäki M, Kärkkäinen M, Lamberg-Allardt C, Laitinen K, Alhava E, Heikkinen J, Impivaara O, Mäkelä P, Palmgren J, Seppänen R, Vuori I, The Cardiovascular Risk in Young Finns Study Group. Exercise, smoking, and calcium intake during adolescence and early adulthood as determinants of peak bone mass. *Br Med J* 1994;309:230-235.
- Välimäki V-V, Moritz N, Yrjans JJ, Vuorio E, Aro HT. Effect of zoledronic acid on incorporation of a bioceramic bone graft substitute. *Bone* 2006;38:432-443.
- Väänänen HK. Osteoclast function: biology and mechanisms. In: Bilezikian JP, Raitz LG, Rodan GA. (Eds.), *Principles in bone biology*. Academic Press, New York, 1996 pp. 103-113.
- Watts NB. Treatment of osteoporosis with bisphosphonates. *Endocrinol Metab Clin North Am* 1998;27:419-439.
- Wilson J, Pigott GH, Schoen FJ, Hench LL. Toxicology and biocompatibility of bioglasses. *J Biomed Mater Res* 1981;15:805-817.
- Winter MC, Coleman RE. Bisphosphonates in the adjuvant treatment of breast cancer. *Clin Oncol (R Coll Radiol)* 2013;25:135-145.
- Woo SB, Hellstein JW, Kalmar JR. Narrative [corrected] review: bisphosphonates and osteonecrosis of the jaws. *Ann Intern Med* 2006;144:753-761.
- Yadav VS, Narula SC, Sharma RK, Tewari s, Yadav R. Clinical evaluation of guided tissue regeneration combined with autogenous bone or autogenous bone mixed with bioactive glass in intrabony defects. *J Oral Sci* 2011;53:481-488.

- Yaffe A, Golomb G, Breuer E, Binderman I. The effect of topical delivery of novel bisacylphosphonates in reducing alveolar bone loss in the rat model. *J Periodontol* 2000;71:1607-1612.
- Yaffe A, Herman A, Bahar H, Binderman I. Combined local application of tetracycline and bisphosphonate reduces alveolar bone resorption in rats. *J Periodontol* 2003;74:1038-1042.
- Yakatan GJ, Poynor WJ, Talbert RL, Floyd BF, Slough CL, Ampulski RF. Clodronate kinetics and bioavailability. *Clin Pharmacol Ther* 1982;31:402-410.
- Yli-Urpo H, Söderling E, Vallittu PK, Närhi T. pH changes induced by bioactive glass ionomer cements. *Key Eng Mater* 2004;599-602.
- Zhou ZH, Yi QF, Nei HD, Ling YL, Zhou JN, Liu LH, Liu XP. Mineralization and osteoblast response to bioactive glass in vitro. *J Med Eng Technol* 2010;34:285-290.